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# EFFECTS OF HYPOPHYSECTOMY AND REPLACEMENT THERAPY ON THE THYROID AND ADRENAL GLANDS OF THE MALE GROUND SQUIRREL[1]

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IT HAS BEEN conclusively demonstrated in recent years that in a variety of laboratory mammals the hypophysis exercises a controlling influence upon the thyroid and adrenal glands. However, no adequate study has been made, to our knowledge, of the role of the pituitary in thyroid and adrenal periodicity as manifested in a wild, annually breeding mammal. The present investigation is designed to fill this gap. The common thirteen-lined ground squirrel of the Middle West, *Citellus tridecemlineatus*, in which various aspects of endocrine relationships have been systematically studied by our laboratories since 1931, constitutes the object of the work reported here. In this species, distinct annual thyroid and adrenal cycles have been observed and described by the senior writer, who has likewise produced experimental modifications of these basic cycles by administration of pituitary extracts (1, 2). The present paper deals, conversely, with the effects of experimental hypopituitarism, achieved by the ablation of the hypophysis, upon the thyroid and adrenal glands of the male ground squirrel.

## MATERIALS AND METHODS

The animals used in this investigation have been previously studied from the viewpoint of pituitary-gonad relationships, and the results of this study have been published elsewhere (3, 4, 5) along with a detailed illustrated description of the technic of hypophysectomy employed as well as with an account of the postoperative care of the animals. The hypophysectomized males were kept alive for periods ranging from 36 to 132 days and killed together with comparable controls.

Aside from hypophysectomized controls (*lot* 1), there were 4 additional lots of animals injected after hypophysectomy with the following preparations: *lot* 2, with androgens; *lot* 3, with gonadin, an extract of blood serum of pregnant mares; *lot* 4, with prospermin; and *lot* 5, with antuitrin-S. The doses and duration of the injection periods are given in the corresponding tables.

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In all hypophysectomized animals, the completeness of pituitary removal was determined by examination of serial sections of the sphenoid bone and of the contents of the sella turcica (3). Only those animals lacking fragments of the pars distalis were included in the present investigation.

At autopsy, the thyroids and adrenals were removed along with the reproductive organs. Both thyroids and both adrenals were weighed fresh. Of each pair of glands, one was fixed in Bouin's fluid and the other in formalin-Zenker.

The tissue was sectioned at 4 to 5 micra and stained with Krichesky's modification of the triple Mallory mixture.

## RESULTS

*Lot 1, hypophysectomized controls.* Considered in this report are 28 hypophysectomized, uninjected animals in which a study of serial sections of the sphenoid region revealed absence of hypophyseal anterior lobe tissue.

Hypophysectomy does not seem to have any appreciable effect on the weight of the thyroid. Although the average weight of the thyroid in the experimental animals is 16.5 mg. while in unoperated controls it amounts to 21.3 mg. this decrease in weight is not significant and is well within the range of normal variability (table 1).

Histologically, the thyroids of the hypophysectomized animals (fig. 1) are characterized by a low epithelium; by an accumulation of abundant, deeply staining intrafollicular colloid; by diminished vascularity; and by an increase in the amount of interfollicular connective tissue.

The annual thyroid cycle consisting of an alternation of a practically inactive phase during the hibernation period (late October to March) and a period of active secretion and colloid resorption during the non-hibernating period (April to late October) is abolished by the removal of the hypophysis. The thyroids of the hypophysectomized animals, as described above, reveal a low degree of physiological activity and resemble the 'resting' thyroids of ground squirrels killed during the period of hibernation.

The adrenal gland undergoes marked atrophy in the absence of the hypophysis. The weight of the adrenals in the hypophysectomized animals is significantly decreased to a mean of 7.1 mg. as compared with the weight of the adrenals in the unoperated controls, which averages 20.7 mg. (table 1). Thus, the average decrease in the weight of the adrenals amounts to 65.7%.

Planimetric measurements of camera lucida tracings of serial sections show that whereas the volume of the adrenal medulla is decreased somewhat following hypophysectomy, it is the cortex which is mainly involved in the adrenal atrophy taking place in the hypophysectomized animal. Thus, the cortex:medulla ratio in a typical hypophysectomized animal amounted to 3.2:1 whereas in a comparable unoperated control it was 8.2:1.

This atrophy of the adrenal following hypophysectomy is evident from histological examination of the adrenal of the hypophysectomized animal (fig. 2). The gland, following preparation for microscopical study, is shrunken, and the capsule is usually thickened and is sometimes thrown into numerous folds due to shrinkage of the entire gland. The cortex is narrow and exhibits dedifferentiation tendencies. The zona fasciculata and 'reticularis A' (1) tend to disappear from the cortex. The zona glomerulosa persists, but its cells appear to be arrested in their development and seem incapacitated for further differentiation. In numerous instances these cells show pathological changes such as shrinkage, clumping of cytoplasmic granules and nuclear pyknosis. The zona fasciculata, in cases where it persists, is narrow and its cells are highly vacuolated. But it is the zona reticularis which exhibits the most extreme signs

TABLE 1. EFFECT OF HYPOPHYSECTOMY ON BODY WEIGHT AND ON WEIGHT OF THYROID AND ADRENAL

| Animal No. | Age[1] | Date of operation | Date killed | Length of hypophysectomy period | Body weight | Weight of thyroids | Weight of adrenals |
|---|---|---|---|---|---|---|---|
| | | | | days | gm. | mg. | mg. |
| | | | *A. Hypophysectomized Animals* | | | | |
| 1 | y | 11.18.35 | 2.15.36 | 89 | 129 | 16.0 | 7.0 |
| 2 | y | 11.18.35 | 2.16.36 | 90 | 170 | 9.8 | 6.4 |
| 3 | y | 11.21.35 | 3.16.36 | 115 | 155 | 11.4 | 5.3 |
| 4 | y | 11.22.35 | 3.16.36 | 114 | 185 | 22.0 | 8.4 |
| 5 | ad | 11.22.35 | 3. 5.36 | 103 | 127 | 24.2 | 7.8 |
| 6 | y | 11.18.35 | 3.25.36 | 128 | 155 | 16.6 | 9.0 |
| 7 | y | 11.20.35 | 3.25.36 | 126 | 210 | 26.0 | 4.8 |
| 8 | y | 11.21.35 | 3.25.36 | 125 | 168 | 14.4 | 5.5 |
| 9 | y | 11.14.35 | 3.25.36 | 132 | 150 | 11.5 | 6.5 |
| 10 | y | 11.21.35 | 3.27.36 | 127 | 155 | 18.3 | 9.0 |
| 11 | y | 10.29.36 | 1.29.37 | 92 | 105 | 10.2 | 8.2 |
| 12 | ad | 11.13.38 | 12.19.38 | 36 | 180 | 13.8 | 8.4 |
| 13 | ad | 11.13.38 | 12.19.38 | 36 | 159 | 20.4 | 9.0 |
| 14 | ad | 11.22.38 | 1.20.39 | 59 | 250 | 21.0 | 9.8 |
| 15 | ad | 11.22.38 | 1.20.39 | 59 | 195 | 13.2 | 4.2 |
| 16 | ad | 11.22.38 | 1.23.39 | 62 | 166 | 11.5 | 7.7 |
| 17 | ad | 11.22.38 | 1.23.39 | 62 | 166 | 19.0 | 10.0 |
| 18 | ad | 11.23.38 | 1.23.39 | 61 | 152 | 17.5 | 5.5 |
| 19 | ad | 11.23.38 | 1.23.39 | 61 | 151 | 11.5 | 6.8 |
| 20 | ad | 11.23.38 | 1.23.39 | 61 | 115 | 15.6 | 5.7 |
| 21 | ad | 11.23.38 | 1.23.39 | 61 | 125 | 16.8 | 6.9 |
| 22 | y | 12.16.38 | 1.24.39 | 39 | 200 | 14.2 | 7.0 |
| 23 | y | 12.16.38 | 1.24.39 | 39 | 197 | 16.2 | 5.8 |
| 24 | y | 12.10.38 | 1.24.39 | 45 | 145 | 16.4 | 4.6 |
| 25 | y | 12.10.38 | 1.24.39 | 45 | 170 | 19.5 | 8.0 |
| 26 | y | 12.10.38 | 1.24.39 | 45 | 220 | 20.2 | 6.8 |
| 27 | y | 12.10.38 | 1.24.39 | 45 | 180 | 18.0 | 6.4 |
| 28 | y | 12.10.38 | 1.24.39 | 45 | 180 | 17.5 | 8.3 |
| Average | | | | | 166 | 16.5 | 7.1 |
| | | | *B. Unoperated, Untreated Controls* | | | | |
| 29 | ad | | 2.15.36 | | 200 | 29.6 | 12.2 |
| 30 | ad | | 3. 5.36 | | 140 | 20.4 | 24.0 |
| 31 | ad | | 3. 5.36 | | 225 | 26.0 | 27.0 |
| 32 | y | | 3. 5.36 | | 125 | 12.2 | 17.0 |
| 33 | ad | | 3. 5.36 | | 225 | 41.0 | 23.0 |
| 34 | ad | | 3.14.36 | | 145 | 13.8 | 16.8 |
| 35 | ad | | 3.14.36 | | 155 | 27.8 | 33.3 |
| 36 | ad | | 3.27.36 | | 190 | 26.3 | 15.6 |
| 37 | ad | | 5. 8.36 | | 165 | 16.4 | 11.5 |
| 38 | ad | | 5.13.36 | | 165 | 11.4 | 21.0 |
| 39 | ad | | 12.24.36 | | 205 | 22.0 | 21.0 |
| 40 | ad | | 1.29.37 | | 190 | 21.5 | 26.8 |
| 41 | ad | | 2.25.37 | | 147 | 15.6 | 34.6 |
| 42 | y | | 1.23.39 | | 200 | 15.8 | 13.0 |
| 43 | ad | | 1.23.39 | | 238 | 20.0 | 13.2 |
| Average | | | | | 181 | 21.3 | 20.7 |

[1] y, young: immature animal which has not reached breeding capacity prior to time of operation. ad, adult: animal which has or has had spermatozoa prior to time of operation.

of deterioration: pyknotic nuclei, fatty infiltration and fibrosis. Large areas, especially in the juxtamedullary layers, have undergone complete destruction and contain mere pyknotic nuclear remnants enmeshed within the network of connective tissue fibers. Of frequent occurrence in the reticular zone are 'giant cells,' each probably consisting of confluent degenerated cells, their cell walls gone and their cytoplasm

transformed into a mass of lipoid granules enclosing a number of nuclear fragments.

The medulla appears normal and healthy although, as mentioned above, it decreases slightly in volume as indicated by planimetric studies.

The annual cycle of the adrenal gland normally parallels the reproductive cycle and is characterized by a highly developed and differentiated cortex during the period

TABLE 2. HYPOPHYSECTOMY FOLLOWED BY ADMINISTRATION OF ANDROGENS

| Animal No. | Age[1] | Date of hypophysectomy | Length of hypophysectomy period | Length of injection period | Daily dose | Date killed | Body weight | Weight of thyroids | Weight of adrenals |
|---|---|---|---|---|---|---|---|---|---|
| | | | *days* | *days* | *mg.* | | *gm.* | *mg.* | *mg.* |
| | | | | *A. Testosterone* | | | | | |
| 44 | ad | 5.29.37 | 184 | 25 | 0.5 | 11.28.37 | 200 | 14.2 | 10.2 |
| 45 | ad | 11.11.38 | 26 | 25 | 0.5 | 12. 7.38 | 155 | 13.5 | 16.4 |
| 46 | ad | 11.11.38 | 31 | 30 | 0.5 | 12.11.38 | 130 | 13.0 | 9.8 |
| 47 | ad | 11.11.38 | 31 | 30 | 0.5 | 12.11.38 | 105 | 13.8 | 10.0 |
| 48 | ad | 11.13.38 | 31 | 30 | 0.5 | 12.13.38 | 115 | 14.0 | 7.8 |
| 49 | ad | 11.18.38 | 29 | 28 | 0.5 | 12.17.38 | 90 | 10.6 | 7.5 |
| 50 | ad | 11.13.38 | 32 | 31 | 0.5 | 12.14.38 | 145 | 16.0 | 6.5 |
| 51 | ad | 11.18.38 | 32 | 31 | 0.5 | 12.19.38 | 126 | 10.2 | 6.8 |
| 52 | ad | 11.19.38 | 31 | 30 | 0.5 | 12.19.38 | 170 | 13.0 | 5.8 |
| 53 | ad | 11.20.38 | 25 | 24 | 0.5 | 12.14.38 | 110 | 12.7 | 4.5 |
| 54 | y | 12.18.38 | 31 | 30 | 0.5 | 1.18.39 | 175 | 17.8 | 4.0 |
| 55 | y | 12.18.38 | 31 | 30 | 0.5 | 1.18.39 | 160 | 13.0 | 4.2 |
| 56 | y | 12.18.38 | 31 | 30 | 0.5 | 1.18.39 | 124 | 26.4 | 8.2 |
| 57 | y | 12.18.38 | 34 | 31 | 0.5 | 1.19.39 | 115 | 8.6 | 5.5 |
| 58 | y | 12.16.38 | 34 | 31 | 0.5 | 1.19.39 | 165 | 12.7 | 6.0 |
| 59 | y | 12.16.38 | 34 | 31 | 0.5 | 1.19.39 | 145 | 17.8 | 4.4 |
| 60 | y | 7.24.36 | 37 | 30 | 1.0 | 8.30.36 | 133 | 14.6 | 7.1 |
| 61 | ad | 11.22.38 | 53 | 26 | 1.0 | 1.14.39 | 115 | 14.6 | 10.0 |
| 62 | ad | 11.22.38 | 55 | 28 | 1.0 | 1.16.39 | 105 | 18.3 | 15.5 |
| 63 | y | 7.24.36 | 37 | 30 | 2.0 | 8.30.36 | 160 | 12.8 | 8.0 |
| Average | | | | | | | 137 | 14.4 | 7.9 |
| | | | | *B. Testosterone Propionate* | | | | | |
| 64 | ad | 5.29.37 | 184 | 25 | 0.5 | 11.28.37 | 150 | 19.2 | 10.2 |
| 65 | ad | 11. 6.38 | 33 | 32 | 0.5 | 12. 9.38 | 160 | 12.8 | 6.2 |
| 66 | ad | 11. 6.38 | 33 | 32 | 0.5 | 12. 9.38 | 190 | 13.8 | 7.0 |
| 67 | ad | 11.23.38 | 51 | 25 | 0.5 | 1.13.39 | 105 | 14.5 | 9.2 |
| 68 | y | 12.18.38 | 30 | 29 | 0.5 | 1.17.39 | 245 | 20.2 | 6.5 |
| 69 | y | 12.18.38 | 31 | 29 | 0.5 | 1.18.39 | 140 | 15.0 | 5.0 |
| 70 | y | 12.18.38 | 31 | 29 | 0.5 | 1.18.39 | 140 | 12.0 | 4.6 |
| 71 | y | 12.18.38 | 31 | 29 | 0.5 | 1.18.39 | 140 | 16.3 | 6.0 |
| 72 | y | 12.18.38 | 31 | 29 | 1.0 | 1.18.39 | 155 | 14.3 | 5.0 |
| 73 | y | 12.18.38 | 31 | 29 | 1.0 | 1.18.39 | 190 | 19.6 | 6.2 |
| Average | | | | | | | 162 | 15.8 | 6.6 |

[1] For explanation of symbols see note 1 under table 1.

of breeding capacity (fig. 3), disappears entirely in the absence of the hypophysis.

*Lot 2, hypophysectomy followed by administration of androgens.* Testosterone or testosterone propionate was administered by subcutaneous injection in daily doses of 0.5 to 2.0 mg. for periods ranging from 24 to 32 days, to 30 hypophysectomized animals.

Neither the thyroid nor the adrenal was affected perceptibly by the treatment. The weights of the thyroids and of the adrenals did not differ significantly from the respective weights in the lot of hypophysectomized controls (table 2.)

The thyroid remained in a physiologically low state of activity, displaying a squamous or low cuboidal epithelium and an abundance of dense stored colloid.

The atrophy of the adrenal resulting from the removal of the hypophysis was not prevented by administration of the androgens employed. The same picture of cortical differentiation and cell destruction prevailed in the adrenals of the hypophysectomized animals injected with testosterone or with its ester.

The medulla was not influenced perceptibly by the treatment.

*Lot 3, hypophysectomy followed by administration of gonadin.* In this series, 7 animals were totally hypophysectomized and injected daily with 0.5 cc. of gonadin for periods ranging from 9 to 32 days.

This treatment resulted in a slight stimulation of the thyroid and in a considerable stimulation of the adrenal.

The weight of the thyroid remained, however, within the normal range of variability whereas the weight of the adrenal showed a substantial increase (table 3) averaging 65.7%.

TABLE 3. HYPOPHYSECTOMY FOLLOWED BY ADMINISTRATION OF GONADIN

| Animal No. | Age[1] | Date of hypophysectomy | Length of hypophysectomy period | Length of injection period | Daily dose | Date killed | Body weight | Weight of thyroids | Weight of adrenals |
|---|---|---|---|---|---|---|---|---|---|
| | | | *days* | *days* | *cc.* | | *gm.* | *mg.* | *mg.* |
| 74 | ad | 7.28.36 | 18 | 16 | 0.5 | 8.15.36 | 170 | 18.0 | 21.0 |
| 75 | y | 7.24.36 | 16 | 10 | 0.5 | 8.19.36 | 118 | 10.8 | 16.0 |
| 76 | y | 7.24.36 | 18 | 12 | 0.5 | 8.11.36 | 125 | 9.7 | 21.8 |
| 77 | y | 7.24.36 | 18 | 12 | 0.5 | 8.11.36 | 117 | 8.8 | 28.4 |
| 78 | y | 7.24.36 | 15 | 9 | 0.5 | 8. 8.36 | 110 | 10.5 | 22.8 |
| 79 | y | 9. 3.36 | 50 | 29 | 0.5 | 10.23.36 | 120 | 11.3 | 12.6 |
| 80 | y | 9. 3.36 | 54 | 32 | 0.5 | 10.27.36 | 110 | 17.9 | 22.1 |
| Average | | | | | | | 124 | 12.4 | 22.1 |

[1] For explanation of symbols see note 1 under table 1.

The thyroid exhibits a low cuboidal epithelium and, in some follicles, a thin, bubbly intrafollicular colloid with few resorption vacuoles.

The adrenal cortex (fig. 4) shows a highly developed fasciculate zone, a wide, greatly stimulated 'reticularis A' subzone and intensive cell destruction in the juxtamedullary layers of the 'reticularis B' subzone,—on the whole, a picture ordinarily ascribed to high secretory activity and to a rapid cell cycle. Numerous hemorrhagic areas may be found in the cortex.

No appreciable effects were observed in the medulla.

*Lot 4, hypophysectomy followed by administration of prospermin.* The number of animals receiving this treatment was 13. The daily dosage varied from 1.0 to 2.5 mg., and the period of injection from 10 to 31 days.

The thyroids showed little or no stimulation. Their weight did not deviate from the normal range (table 4). The follicular epithelium remained low and apparently inactive, with dense colloid stored intrafollicularly. Only in 2 cases was the colloid thin and bubbly.

Adrenal atrophy was, however, prevented by the treatment. The weight of the adrenals increased to within the range for normal animals, 17.4 mg. as compared with 7.1 mg. in the hypophysectomized controls. The histological structure of the gland was maintained at a normal or slightly subnormal level and was typical of the male

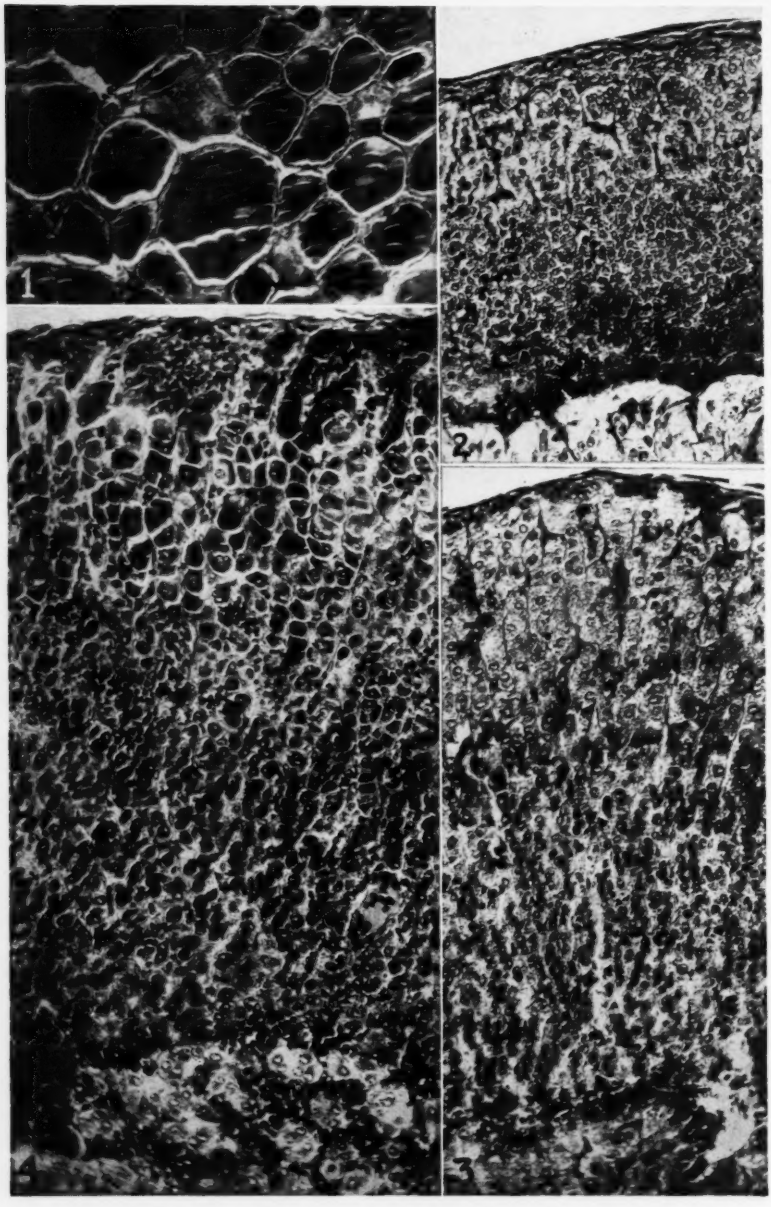


Fig. 1. THYROID OF A HYPOPHYSECTOMIZED ANIMAL, PITUITARY-LESS FOR 45 DAYS. Note the low epithelium and the abundance of deeply staining, dense stored colloid. Formol-zenker; Krichesky-Mallory; 5 $\mu$; × 120. Fig. 2. ADRENAL OF HYPOPHYSECTOMIZED ANIMAL, PITUITARY-LESS FOR 61 DAYS. The narrow cortex consists essentially of two zones; zona glomerulosa and 'reticularis B' (inner reticular). Formol-zenker; Krichesky-Mallory; 5 $\mu$; × 200. Fig. 3. ADRENAL OF A NORMAL MALE CONTROL DURING THE SEASON OF

TABLE 4. HYPOPHYSECTOMY FOLLOWED BY ADMINISTRATION OF PROSPERMIN

| Animal No. | Age[1] | Date of hypophysectomy | Length of hypophysectomy period | Length of injection period | Daily dose | Date killed | Body weight | Weight of thyroids | Weight of adrenals |
|---|---|---|---|---|---|---|---|---|---|
| | | | *days* | *days* | *mg.* | | *gm.* | *mg.* | *mg.* |
| 81 | y | 10.29.36 | 92 | 31 | 1.5 | 1.29.37 | 145 | 15.5 | 12.0 |
| 82 | y | 12.11.36 | 28 | 10 | 1.0 | 1. 8.37 | 106 | 17.0 | 110.0 |
| 83 | y | 12.11.36 | 35 | 18 | 1.0 | 1.15.37 | 120 | 18.4 | 22.2 |
| 84 | y | 12.11.36 | 30 | 13 | 0.5 | 1.10.37 | 97 | 20.7 | 16.6 |
| 85 | y | 10.22.37 | 30 | 18 | 2.5 | 11.21.37 | 109 | 19.2 | 11.5 |
| 86 | ad | 5.29.37 | 177 | 20 | 2.5 | 11.22.37 | 140 | 12.3 | 12.2 |
| 87 | y | 10.22.37 | 34 | 23 | 2.5 | 11.25.37 | 85 | 13.5 | 14.4 |
| 88 | ad | 5.29.37 | 183 | 25 | 2.5 | 11.28.37 | 140 | 19.2 | 20.2 |
| 89 | ad | 5.29.37 | 183 | 25 | 2.5 | 11.28.37 | 120 | 16.5 | 22.4 |
| 90 | ad | 5.29.37 | 183 | 25 | 2.5 | 11.28.37 | 145 | 16.5 | 13.6 |
| 91 | y | 10.20.37 | 39 | 25 | 2.5 | 11.28.37 | 150 | 17.0 | 17.7 |
| 92 | y | 5.29.37 | 183 | 25 | 2.5 | 11.28.37 | 180 | 23.8 | 11.2 |
| 93 | y | 10.22.37 | 37 | 25 | 2.5 | 11.28.37 | 135 | 16.1 | 11.6 |
| Average | | | | | | | 129 | 17.4 | 15.0 |

[1] For explanation of symbols see note 1 under table 1.

ground squirrel during the period of aspermia. The glomerulous, fasciculate and reticular zones of the cortex were distinct, but there was no differentiation in the reticular zone. No observable changes were found in the medulla.

*Lot 5, hypophysectoy followed by administration of antuitrin-S.* In the 6 animals of this series, injected subcutaneously following hypophysectomy with 25 to 100

TABLE 5. HYPOPHYSECTOMY FOLLOWED BY ADMINISTRATION OF ANTUITRIN-S

| Animal No. | Age[1] | Date of hypophysectomy | Length of hypophysectomy period | Length of injection period | Daily dose | Date killed | Body weight | Weight of thyroids | Weight of adrenals |
|---|---|---|---|---|---|---|---|---|---|
| | | | *days* | *days* | R.U. | | *gm.* | *mg.* | *mg.* |
| 94 | y | 7.17.36 | 29 | 16 | 100 | 8.15.36 | 105 | 6.6 | 18.3 |
| 95 | y | 7.24.36 | 18 | 12 | 100 | 8.11.36 | 109 | 12.8 | 22.9 |
| 96 | y | 7.24.36 | 15 | 9 | 100 | 8. 8.36 | 117 | 10.2 | 16.2 |
| 97 | ad | 8. 6.36 | 16 | 12 | 25 | 8.27.36 | 126 | 8.5 | 14.6 |
| 98 | y | 7.17.36 | 69 | 42 | 25 | 9.24.36 | 105 | 16.0 | 11.7 |
| 99 | y | 9. 3.36 | 52 | 30 | 50 | 10.25.36 | 110 | 16.1 | 15.8 |
| Average | | | | | | | 112 | 11.7 | 16.5 |

[1] For explanation of symbols see note 1 under table 1.

units of antuitrin-S for 9 to 42 days, the thyroid remained unstimulated. It was a typically 'resting' gland, with its characteristically low epithelium and intrafollicular accumulation of deeply staining colloid. The weight of the thyroid, averaging 11.7 mg., showed no significant deviation from the mean of 16.5 mg. for the hypophysectomized controls.

The adrenal, on the other hand, underwent considerable hypertrophy. Its average weight in this group amounted to 16.5 mg. and showed an increase of 56.9% over the mean of 7.1 mg. of the hypophysectomized controls.

BREEDING CAPACITY. Note the wide, highly differentiated cortex and the well developed fasciculata and 'reticularis A.' Formol-zenker; Krichesky-Mallory; 5 $\mu$; × 200. Fig. 4. ADRENAL OF A HYPOPHYSECTOMIZED ANIMAL INJECTED WITH 0.5 CC. GONADIN DAILY FOR 32 DAYS. Note the tremendous development of the cortex and the extreme expansion of the 'reticularis A' at the expense of the fasciculate and glomerulous zones. Formol-zenker; Krichesky-Mallory; 5 $\mu$; × 200.

The adrenal cortex presented unmistakable evidence of a high degree of anatomical and physiological stimulation: a wide fasciculate zone consisting of large cells with a highly granular cytoplasm; a clear-cut differentiation of the reticular zone into a wide, conspicuous 'reticularis A' and a similarly expanded 'reticularis B' subzone studded with degenerating cells which have probably completed a rapid, active cycle. The medulla appeared normal.

## DISCUSSION

In this investigation, evidence was presented to the effect that in the male of the annually breeding rodent *Citellus tridecemlineatus* the hypophysis controls largely, if not wholly, the annual cycles of the thyroid and adrenal glands. In the absence of the hypophysis, the thyroid remains an essentially 'resting' gland throughout the year, and the adrenal cortex undergoes considerable atrophy and degeneration.

It is interesting to note that the quiescent hypophysectomy thyroid resembles essentially the normal 'resting' gland during the hibernation period. Apparently, under normal conditions, the hypophysis is thyrotropically inactive or slightly active during this period.

The weight of the thyroid does not change significantly following hypophysectomy. There is a slight decrease in the average weight of the hypophysectomy thyroid but in view of the diminished body weight following hypophysectomy, this apparent decrease in the weight of the thyroid cannot be considered as an indication of atrophy. Moreover, the range of variability in the weight of the thyroid in the normal ground squirrel at any given period of the year is so wide that it would take a much larger series of experimental animals and comparable controls than that included in the present investigation to establish the statistical significance of a possible decrease in the weight of the thyroid following hypophysectomy. We must also bear in mind that in the case of the ground squirrel, the weight of the thyroid, unless strikingly changed in magnitude, is an unsatisfactory criterion of physiological activity of the gland: flattening of the epithelium is usually associated with greater accumulation of intrafollicular colloid and increase in stroma. Thus, the decrease in the volume of parenchyma is compensated by the increased mass of colloid and connective tissue, and the total weight of the thyroid may remain unchanged despite physiological changes which may occur within the gland.

Although the thyroid gland of the hypophysectomized ground squirrel closely resembles in histological appearance the thyroid of the hypophysectomized rat as described by Smith (6), the thyroid of the ground squirrel does not seem to undergo actual atrophy following hypophysectomy as unquestionably demonstrated and reported by Smith for the rat. It merely returns to the low winter state and remains in this condition.

The striking decrease in the weight of the adrenal following hypophysectomy is due largely, if not wholly, to the atrophy of the cortex. The medulla does not seem to change in structure. These results are in complete accord with the findings in the rat (6–9).

In contrast to the thyroid, the adrenal cortex, in the absence of the hypophysis, not only returns to its inactive winter phase, but actually undergoes retrogression and degeneration. The fasciculate zone is narrow, shows pathological changes and in some cases disappears from the cortex. The outer reticular subzone disappears entirely. It will be recalled (1) that the outer reticular subzone, termed "reticularis A," is a transitory layer suppressed in the male during the period of aspermia and developed to its greatest extent during the breeding period.

It seems therefore that cyclic adrenotropic influences emanating from the pituitary gland are primarily responsible for the seasonal fluctuations of this subzone in the normal animal. It may be suggested further that the fasciculate zone and the 'reticularis A' subzone are functionally the essential regions of the cortex. The irreversible cell cycle in the adrenal cortex would thus appear as follows: Glomerulosa→Fasciculata→Reticularis A→Reticularis B, where the zona glomerulosa represents the zone of primordial cells; the zona fasciculata, the zone of active secretory cells; the 'reticularis A' subzone, a region of highly specialized cells whose function is related in some manner to reproductive activity; and the 'reticularis B' subzone, a region of senescent, moribund elements. The fact that cell destruction is most prominent in the 'reticularis B' subzone, further supports the hypothesis that this region consists of declining cells.

In the presence of the hypophysis, this cell cycle is complete, and is apparently accomplished by inward migration and differentiation. During the period of reproductive capacity, certain hormonal influences proceeding from the pituitary prolong and stimulate the 'reticularis A' phase of the cortical cell. In the absence of the hypophysis the glomerulosa cell apparently migrates inward, is transformed into an abbreviated fasciculata phase or directly into a 'reticularis B' cell and degenerates rapidly without passing through the active functional phases of the well developed fasciculate and 'reticularis A' cells.

Testosterone and testosterone propionate were ineffective either in stimulating the thyroid or in preventing the atrophy of the adrenal cortex of the hypophysectomized animal. These androgens did not, therefore, replace the thyrotropic and adrenotropic hormones of pituitary origin in the ground squirrel.

Gonadin and, to a slight extent, prospermin stimulated the hypophysectomy thyroid. This effect may be due to traces of thyrotropic substances present in these preparations. Antuitrin-S did not affect the thyroid of the hypophysectomized animal.

The atrophied adrenal cortex of the hypophysectomized male was restored to the normal state and even stimulated in descending order of effectiveness by gonadin, antuitrin-S and prospermin. Apparently, these substances contain adrenotropic fractions. Whether or not these fractions are identical with the adrenotropic hormone or hormones normally secreted by the hypophysis, is, of course, unknown. It is noteworthy, however, that the adrenal cortex of the animals injected with these gonadotropic preparations contained such pathological features as extensive hemorrhagia and intensive cell destruction. These pathological phenomena were probably produced by toxic influences of the materials injected as evidenced by the decline in physical vigor and death of some of the animals treated with the substances in question. That toxins may have similar effects upon the adrenal cortex was demonstrated earlier by Hoerr (10).

## SUMMARY

Hypophysectomy in the male ground squirrel (*Citellus tridecemlineatus*) abolishes the annual cycle of the thyroid. The thyroid of hypophysectomized males shows histological evidence of low physiological activity and resembles the thyroid of ground squirrels during the hibernating period.

The adrenal of the hypophysectomized male ground squirrel undergoes marked atrophy, particularly evident in the cortex. The fasciculate and outer reticular zones disappear, and degenerative changes become prominent in the cortex, especially in the dedifferentiated reticular zone.

Administration of testosterone and testosterone propionate by subcutaneous injection to hypophysectomized males failed to stimulate the thyroid or to prevent the atrophy of the adrenal.

Of the gonadotropic substances injected subcutaneously into hypophysectomized males, gonadin stimulated slightly the thyroid and not only prevented the involution of the adrenal, but also resulted in cortical hypertrophy. Antuitrin-S and prospermin prevented adrenal atrophy and stimulated the adrenal cortex, but had no effect on the hypophysectomy thyroid.

The pituitary appears therefore to be necessary for the normal functioning of the thyroid and adrenal of the male ground squirrel (*Citellus tridecemlineatus*) and for the maintenance of the annual cycles of these glands in this species.

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# THE EFFECTIVENESS OF PRIMING DOSES OF DESOXYCORTICOSTERONE ACETATE IN PROTECTING THE ADRENALECTOMIZED DOG AGAINST WATER INTOXICATION

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THE SEQUENCE of symptoms in the response of an animal to the continued forcing of water have been well described (1–4). The major features are a progressive excitability, salivation, and finally severe convulsions with opisthotonus. These symptoms are believed linked in some way with intracellular hydration, the terminal convulsions being attributed to a cerebral edema. Fatal intoxication in the normal animal is difficult to produce, for large amounts of water must be frequently given over long periods of time, and the cessation of water administration even when convulsions have ensued is usually followed by spontaneous recovery. In the adrenalectomized animal, however, a relatively small quantity of water will produce convulsions, and spontaneous recovery is never manifested (5–7). An intravenous injection of large amounts of cortical extract or hypertonic salt is required to save the life of the animal (5). Since the adrenalectomized animal primed with cortical extract previous to the administration of water responds in every respect as does the intact animal, this susceptibility to water intoxication is a reflection of some direct action of the hormone or hormones, contained in the extract itself.

The synthetic steroid, desoxycorticosterone acetate, has been successfully used for replacement therapy in uncomplicated adrenal insufficiency (8–12) and as protection against shock as a result of trauma (13). In these experiments, its ability to replace cortical extract in this protective action against water intoxication has been tested.

## METHODS

The adrenalectomized dogs employed as test animals were strong, healthy, and at peak weight. They had been maintained for at least 4 months on adequate daily injections of cortical extract. The control unoperated dogs were of approximately the same size and weight. In one control dog, the kidneys were removed, one 7 days, and the other 3 hours, before the experiment was begun.

Priming with cortical extract was by the intravenous injection of 3 cc. per kg. body weight just prior to the start of the experiment. Priming with D.C.A. (desoxycorticosterone acetate)[2] was by 3 intramuscular injections of 5 or 10 mg. each at 18, 12, and 2 hours before the experiment was begun. The dogs were placed in metabo-

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[2] We are indebted to the Ciba Pharmaceutical Products, Inc. for generous supplies of the desoxycorticosterone acetate (percorten) used in these experiments.

lism cages overnight; the next morning, after the bladder had been voluntarily emptied, distilled water, in doses of 25 cc. per kg. body weight was given hourly by stomach sound. When possible, any vomitus or water regurgitated during the experiment was strained and readministered in small amounts with succeeding doses of water.

## RESULTS

*A) Intact dog.* The rate of water administration used was not sufficient to evoke convulsions in more than about 50% of the intact dogs used. At least when, after about the twelfth dose of water, the hourly urine production still balanced the fluid intake, and no symptoms of an approaching intoxication were present, the experiment was discontinued. Those animals in which convulsions were induced required about 10 doses of water and promptly recovered following cessation of water administration.

A quantitative study of the amount of water required to produce intoxication in the dogs used must be inaccurate, since there was no measure of the fluid in the intestinal tract still unabsorbed, and the urine in the bladder unvoided, at the time of convulsions. Since this factor was present in all groups of dogs, no corrections were deemed necessary. Calculations based on serum and urine electrolyte concentrations indicated that the water administered was probably distributed over the whole of the body fluid, diluting the intracellular and extracellular compartments proportionately. The extent of the overall intracellular hydration can therefore be directly calculated from the balance data. Various tissues of the body may, of course, have varied in the amount of water taken up (4).

Table 1 shows that the intact dog will go into convulsions when the water retained is of the order of 140 cc. per kg. body weight. This represents an intracellular hydration of 121%. The retention was lower in those animals in which severe vomiting had occurred, and all vomitus was not replaced. The chain of symptoms which precede actual convulsions became apparent when the intracellular hydration was in the neighborhood of 117%.

A completely nephrectomized dog went into convulsions at about the same fluid retention as the intact dog. The mere presence or absence of urine flow, therefore does not influence appreciably the intracellular hydration level at which convulsions occur. The variable between dogs would appear to be the relative abilities of the kidneys to excrete the excess water and keep the volume of body fluids below the toxic level.

*B) Adrenalectomized dog, untreated.* Not over 6 water administrations of 25 cc. per kg. body weight were usually required to induce convulsions in the untreated adrenalectomized dog. The primary deficiency is a renal failure, for less than a third as much urine was excreted as in the intact animals. Some dogs remained anuric throughout the experiment. This failure of proper excretion obviously allows the more rapid dilution of the intracellular compartment to the level at which convulsions appear. The reasons for this failure of the kidney to eliminate the excess water are not clear. There is seemingly no relation to the known renal deficiency which allows an electrolyte and water diuresis in uncomplicated adrenal insufficiency. The tendency toward salt diuresis was present in the adrenalectomized dogs used in these experiments also, since the total electrolyte excretion was greater than that of the intact animals (5). A commensurate amount of water was not excreted, however. The renal failure leads to a more rapid dilution of the serum electrolyte concentrations than in the intact animal (5), but the concentrations never reach a level which would, under normal conditions, prevent proper urine formation in either intact or adrenalectomized animal.

TABLE 1. WATER BALANCES IN DOGS AT THE TIME OF THE FIRST CONVULSION PRODUCED BY THE CONTINUED FORCING OF WATER

| Adrenalectomized, no extract | | | | Intact and primed adrenalectomized[1] | | | |
|---|---|---|---|---|---|---|---|
| Dog | Water given | Urine volume | Water retention | Dog | Water given | Urine volume | Water retention |
| | cc./kg. | cc./kg. | cc./kg. | | cc./kg. | cc./kg. | cc./kg. |
| 1 | 105 | 17 | 88 | Intact, normal controls | | | |
| 2 | 70 | 9 | 61[2] | | | | |
| 3 | 150 | 68 | 82 | 12 | 300 | 160 | 140 |
| 4 | 125 | 32 | 93 | 13 | 325 | 182 | 143 |
| 5 | 125 | 18 | 107 | 14 | 225 | 115 | 110[2] |
| 6 | 140 | 34 | 106 | 15 | 303 | 173 | 130 |
| 7 | 129 | 0 | 129[3] | 16 | 160 | 50 | 110[2] |
| 8 | 128 | 0 | 128[3] | 17 | 197 | 55 | 142 |
| 9 | 118 | 0 | 118 | Nephrectomized | | | |
| 10 | 93 | 0 | 93 | | | | |
| 11 | 135 | 44 | 91[2] | 18 | | | 131 |
| | | | | Adrx., primed with D.C.A. | | | |
| | | | | 19 | 194 | 85 | 109[2] |
| | | | | 20 | 275 | 127 | 148 |
| | | | | 21 | 225 | 70 | 155 |
| | | | | 22 | 225 | 65 | 160 |
| | | | | Adrx., primed with cortical extract | | | |
| | | | | 23 | 407 | 239 | 168 |
| Av. | 120 | 20 | 100±4.1 | Av. | 254 | 116 | 137±3.9 |

[1] Intact and primed adrenalectomized dogs are grouped together since they respond in similar fashion to water administration.
[2] Excess vomiting, all vomitus not replaced.
[3] High values not confirmed by electrolyte analyses.

A second factor is present in the greater susceptibility of the adrenalectomized dog to water intoxication, for, as table 1 clearly shows, the fluid retention at which convulsions are induced is significantly lower than in the intact dog. Intracellular hydration at the time of convusions is of the order of 115%, a level actually lower than that at which symptoms first appear in the intact dog. Electrolyte analyses have revealed no evidence of an imbalance between the dilution of the two compartments of

TABLE 2. EFFECT OF PRIMING WITH D.C.A. ON WATER INTOXICATION IN THE ADRENALECTOMIZED DOG

| Dog | Dose | Water given | Urine volume | Water retention | Remarks |
|---|---|---|---|---|---|
| | mg. | cc./kg. | cc./kg. | cc./kg. | |
| 1 | 15 | 150 | 48 | 102 | Some salivation, sway |
| 2 | 15 | 150 | 49 | 101 | No symptoms |
| 3 | 15 | 150 | 18 | 132 | Salivation, excitable |
| 4 | 15 | 150 | 50 | 100 | No symptoms |
| 5 | 15 | 150 | 87 | 63 | No symptoms |
| 6 | 15 | 150 | 70 | 80 | No symptoms |
| 7 | 15 | 150 | 77 | 73 | No symptoms |
| 8 | 30 | 150 | 37 | 113 | No symptoms |
| 9 | 30 | 150 | 26 | 124 | No symptoms |
| 10 | 30[1] | 150 | 46 | 104 | No symptoms |
| 11 | 30[1] | 150 | 39 | 111 | Salivation, excitable |
| Av. | | 150 | 50 | 100 | |

[1] 'Aqueous' D.C.A., see text.

the body, so that this difference must indicate a greater sensitivity to hydration in the adrenalectomized dog.

C) *Adrenalectomized dog, primed with cortical extract.* Priming with cortical extract restores both the kidney factor and the "sensitivity to hydration" to normal. Only one of the 3 adrenalectomized dogs so primed could be driven into convulsions, and this one required 16 water administrations. The rate of urine production was entirely normal.

D) *Adrenalectomized dog, primed with D.C.A.* D.C.A., as cortical hormone, restored both deficiencies to normal. Only 4 of 9 dogs primed with D.C.A. in oil went into convulsions, and these took from 8 to 11 water administrations. Since intoxication in the intact dog may be produced in some animals with 9 to 10 water administrations, the experiments on the other 5 dogs were not carried beyond 9 hours. At this time no symptoms were present. For comparison with the nonprimed adrenalectomized dogs, the fluid balance and symptoms at the end of the sixth water administration, the maximum required to produce convulsions in the untreated dogs, are given in table 2. Two animals only showed symptoms at this stage. The actual retention volumes were often greater at this time than that which leads to convulsions in the untreated dogs. The rate of urine production, although it may be less than in the intact dog, is still two and a half times greater than in the untreated animals.

A doubling of the D.C.A. dosage to 30 mg. gave no demonstrable change in the resistance to the forcing of water. The 15 mg. dose must, therefore, be entirely adequate. But poor success has been obtained with the use of D.C.A. dissolved in an aqueous medium.[3] It did protect two dogs when given in priming doses (table 2) but it would not revive 3 untreated dogs which were allowed to go into convulsions. It was necessary to use cortical hormone to save the lives of these animals. It is believed that the D.C.A. in this solution recrystallized in the blood stream, and hence all injected was not available to the animal. The fact that it did offer protection in some cases is perhaps evidence that even a smaller amount of D.C.A. than the 15 mg. dose would be ample.

## SUMMARY

The adrenalectomized dog develops water intoxication convulsions after the retention of significantly less water than does the intact animal.

The kidney of the adrenalectomized dog cannot excrete the excess water at a normal rate, a deficiency seemingly not related to electrolyte deficiencies.

Desoxycorticosterone acetate will restore to normal both the kidney factor and the "sensitivity to hydration" factor.

Priming doses of desoxycorticosterone acetate, therefore, protects the adrenalectomized dog against water intoxication.

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# SODIUM AND CHLORIDE BALANCE IN CATS AS AFFECTED BY DIABETES INSIPIDUS, ADRENAL INSUFFICIENCY, AND PITRESSIN INJECTIONS

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IT HAS PREVIOUSLY BEEN SHOWN (1, 2) that the usual post-adrenalectomy changes in serum sodium and chloride do not occur in cats with diabetes insipidus, unless pitressin is injected. Since it has been suggested (3–5) that changes in plasma electrolytes in adrenal insufficiency may be secondary to abnormal excretion of these substances, it becomes of interest to determine whether or not there is any difference between polyuric and non-polyuric animals in their excretion of electrolytes after adrenalectomy. We have sought to obtain evidence regarding a possible role of the posterior lobe of the pituitary in electrolyte metabolism, both in the presence and absence of the adrenals, and the present paper, accordingly, is concerned with the excretion of sodium and chloride in diabetes insipidus, adrenal insufficiency, and in the presence of both syndromes combined, together with the effect of pitressin injections on electrolyte excretion in these conditions.

## METHODS

The methods used for care of the cats and for producing diabetes insipidus have been referred to elsewhere (2). Urine sodium was determined by the procedure of Butler and Tuthill (6). For the determination of chloride in cat urine, we found it essential for accurate results to use a higher thiocyanate : $AgNO_3$ ratio than that usually employed for chloride in human urine; also, it is necessary to digest cat urine. The method which Wilson and Ball (7) described for serum chloride was found to be satisfactory, except that the size of the urine sample taken had to be varied according to the degree of polyuria evidenced by the animal.

The animals were kept in metabolism cages and aliquots of 24-hour urine samples were used for analysis. The cats were not catheterized, because it was found that the procedure of catheterization produced such a strong emotional disturbance that the flow of urine was inhibited, both in the normal and in the diabetes insipidus (hereafter called d.i.) animals. It is recognized that collecting the urine of voluntary micturition introduces the possibility of error in any given 24-hour period, but in the d.i. cats with large urine volumes, this error must be slight, and even in the normal animals the errors cancel out over a period of observation of several days duration.

## OBSERVATIONS

*Electrolyte excretion in normal and in untreated d.i. cats.* Before comparing the effect of adrenalectomy on sodium and chloride excretion in normal and in d.i. animals, it is important to ascertain whether uncomplicated d.i. modifies the electrolyte

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excretion, and whether our technic is successful in obtaining accurate electrolyte balances under ordinary conditions. Figure 1 shows the changes which occur in chloride excretion during the development of experimental d.i. Coinciding with the sudden onset of the transient polyuria which follows the operation, there is a sharp increase in chloride excretion. The chloride 'spilling' generally lasts for only one day, or at the most, two days, and is followed usually by some degree of chloride retention to make up at least in part for the chloride which was lost during the first day of the polyuria. A fairly good balance between chloride intake and output is usually established by the end of the interphase between the transient and permanent poly-

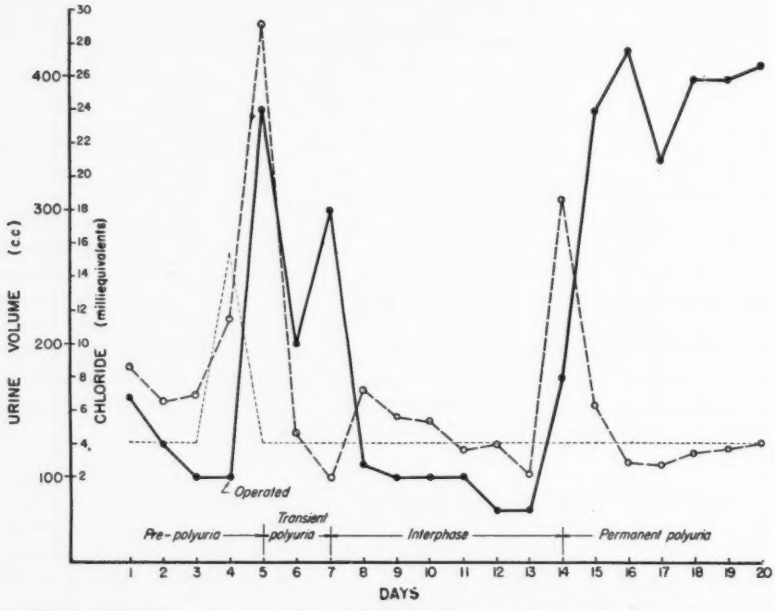


Fig. 1. CHLORIDE EXCHANGE AND URINE VOLUME DURING THE DEVELOPMENT OF THE POLYURIA OF EXPERIMENTAL DIABETES INSIPIDUS IN A CAT. Solid line, urine volume; broken line, chloride output; dotted line, chloride intake; 100 cc. of 0.9% NaCl was injected at the time of the operation.

urias, but when the permanent polyuria is sudden in onset, there is again a marked increase in chloride excretion on the first day of the polyuria. Although these sudden changes in chloride output appear coincidentally with apparent changes in the functional capacity of the posterior lobe, it does not necessarily follow that the unusually large chloride output is a direct effect of a sudden alteration in posterior lobe function. We are inclined to regard it rather as incidental to the sharp increase in urine volume. The result shown in figure 1 is typical of 5 out of 6 cats whose chloride excretion we have followed during the period of the establishment of the polyuria; the sixth cat was atypical in that the polyuria developed gradually—in this case, there was no sudden increase in urine volume, and no 'spilling' of chlorides. That the chloride 'spilling' is indeed due to a sudden increase in urine output is further indicated by the fact that the same phenomenon can be observed under other conditions when the urine volume is suddenly increased. Figure 2 is an example of such a case. A cat with well established polyuria and good balance between chloride intake and output was

deprived of water for a period of 4 days; during this time, the urine volume dropped to a comparatively low figure. When the animal was again allowed free access to water, there was a sudden increase in the urine volume, and a sharp peak in the chloride excretion, just as was observed at the onset of the polyuria. Experiments on 2 other cats gave similar results.

It would seem, then that the chloride 'spilling' is a result of the sudden increase in water output, and is not directly related to posterior lobe function. Priestley (8) noted that water diuresis in man is accompanied by a transient increase in chloride output, and Strauss (9) found that when large quantities of water were drunk there

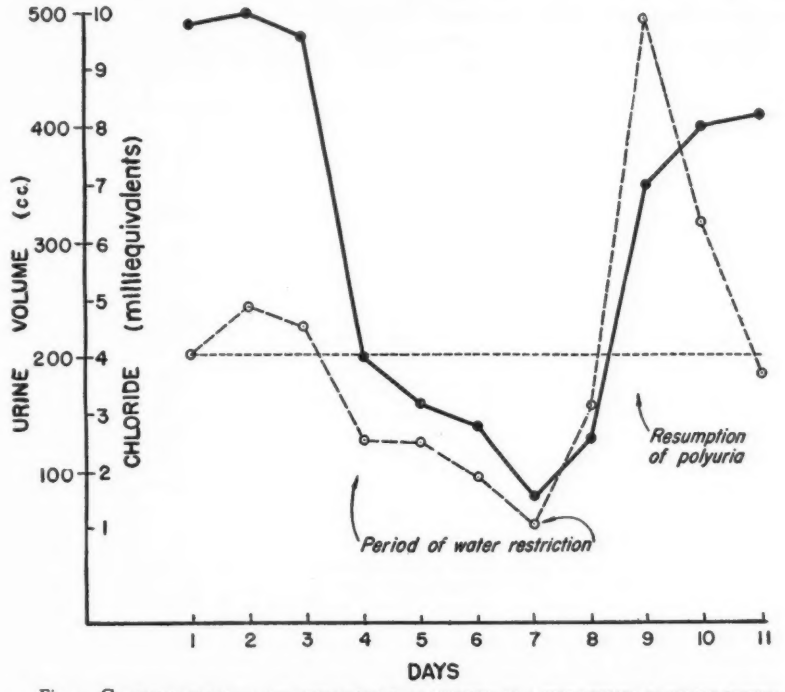


Fig. 2. CHLORIDE EXCHANGE AND URINE VOLUME AS AFFECTED BY A SHORT PERIOD OF WATER RESTRICTION IN A CAT WITH DIABETES INSIPIDUS. During the restricted period, the cat received its usual meat and milk, but was allowed no water. Symbols are same as in figure 1.

was an increase in the daily output of chloride in the urine. Eggleton (10) stated that in dilution diuresis in the anesthetized dog there is an increase in chloride clearance. Rehberg (11) observed that in man, when the plasma chloride exceeds 106 millimols per liter, the chloride excretion rate is affected by the rate of urine formation; the greater the urine volume, the larger the fraction of filtered chloride escaping reabsorption; the serum chloride level in our cats is generally of the order of 116 millimols per liter.

Table 1 shows that after the polyuria is well established, the presence of diabetes insipidus does not alter the daily salt balance in the cat on a constant diet. It is evident that in both the normal and the d.i. animals, ingested sodium and chloride may be quantitatively recovered in the urine under ordinary conditions. On the basis of

these figures, it seems safe to assume that a consistently observed imbalance of sodium or chloride amounting to more than 1 m. eq. daily would reflect a significant alteration from normal in the salt metabolism.

*Effect of pitressin injections on sodium and chloride exchange in normal and d.i. cats.* Many investigators (12–22) have pointed out that the injection of a comparatively large single dose of posterior pituitary extract will bring about an acute increase in chloride excretion. However, we wished to determine the effect on sodium and chloride exchange of a prolonged series of comparatively small doses, such as are used in the control of the polyuria of diabetes insipidus. We have used 3 pressor units per injection; from our experience, this appears to be not far from the minimal amount required to control the polyuria of diabetes insipidus in the cat, when given subcutaneously 3 times daily. A protocol of one experiment is given in table 2. It is to be noted that sodium and chloride balances continue to be negative for about a

TABLE 1. SALT EXCHANGE IN NORMAL AND IN DIABETES INSIPIDUS CATS ON CONSTANT DIET

| Kind of cats | Cat No. | Daily Cl exchange | | | | Daily Na exchange | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Days observed | Av. intake | Av. output | Av. balance | Days observed | Av. intake | Av. output | Av. balance |
| | | | *m.eq.* | *m.eq.* | *m.eq.* | | *m.eq.* | *m.eq.* | *m.eq.* |
| Diabetes insipidus | 1 | 24 | 4.07 | 4.16 | −0.09 | 11 | 5.18 | 5.05 | 0.13 |
| | 2 | 17 | 4.07 | 4.22 | −0.15 | 6 | 5.18 | 4.83 | 0.35 |
| | 3 | 28 | 4.07 | 3.95 | 0.12 | 9 | 5.18 | 5.68 | −0.50 |
| | 4 | 22 | 3.66 | 3.65 | 0.01 | 3 | 5.18 | 6.78 | −1.60 |
| | 5 | 14 | 4.07 | 3.88 | 0.19 | 5 | 5.18 | 4.78 | 0.40 |
| | 6 | 14 | 4.07 | 3.88 | 0.19 | 6 | 5.18 | 5.57 | −0.39 |
| | 7 | 10 | 4.07 | 4.00 | 0.07 | 7 | 5.18 | 3.98 | 1.20 |
| | 8 | 7 | 4.07 | 4.43 | −0.36 | 7 | 5.18 | 4.78 | 0.40 |
| | Average | | 4.02 | 4.02 | 0.00 | | 5.18 | 5.18 | 0.00 |
| Controls | 9 | 9 | 4.07 | 3.98 | 0.09 | 9 | 5.18 | 4.41 | 0.77 |
| | 10 | 27 | 4.07 | 3.80 | 0.27 | 3 | 5.18 | 4.85 | 0.33 |
| | 11 | 21 | 4.07 | 4.00 | 0.07 | 6 | 5.18 | 4.39 | 0.79 |
| | 12 | 14 | 4.27 | 4.13 | 0.14 | 3 | 5.18 | 4.68 | 0.50 |
| | 13 | 21 | 4.07 | 3.94 | 0.13 | 11 | 5.18 | 5.19 | −0.01 |
| | 14 | 15 | 4.07 | 3.75 | 0.32 | 3 | 5.18 | 5.54 | −0.36 |
| | 15 | 5 | 4.07 | 4.16 | −0.09 | 3 | 5.18 | 6.22 | −1.04 |
| | 16 | 3 | 4.07 | 4.38 | −0.21 | 2 | 5.18 | 4.55 | 0.63 |
| | 17 | 20 | 4.01 | 3.77 | 0.24 | 6 | 5.18 | 4.72 | 0.46 |
| | Average | | 4.09 | 3.99 | 0.10 | | 5.18 | 4.95 | 0.23 |

week during the administration of pitressin. There is then a compensatory retention of salt until a good balance between intake and output is once more established, and this occurs whether pitressin injections are continued or not. During the period of negative salt balance, sodium and chloride appear to be lost in equivalent amounts. As the injections are continued, the salt output becomes so adjusted that in the long run the salt balance does not differ from that of an uninjected animal.

Table 3 gives a comparison of the responses of normal and of d.i. cats to courses of pitressin treatment during the period when the effect of the extract on salt excretion is greatest; i.e., during the first 3 to 4 days. It is clear that salt excretion is much less markedly affected in the d.i. than in the unoperated animals, and a negative salt balance is not invariably induced by pitressin in the former. This is in marked contrast to the relative sensitivity of the water exchange mechanism of the 2 groups, for pitressin has a much greater effect on the water excretion of d.i. animals than on that of normal cats. D.i. cats respond promptly to pitressin by a disappearance of the polyuria and polydipsia; this effect lasts as long as the pitressin injections are con-

TABLE 2. EFFECT OF PITRESSIN INJECTIONS UPON SALT AND WATER EXCHANGE OF A NORMAL CAT

| Date | Pitressin injected, pressor units | Urine volume | Cl exchange | | | Na exchange | | |
|---|---|---|---|---|---|---|---|---|
| | | | intake | output | balance | intake | output | balance |
| | | cc. | *m.eq.* | *m.eq.* | *m.eq.* | *m.eq.* | *m.eq.* | *m.eq.* |
| 7/31 | 0 | 90 | 4.07 | 3.43 | 0.64 | 5.18 | 5.60 | −0.42 |
| 8/ 1 | 0 | 100 | 4.07 | 4.93 | −0.86 | 5.18 | 6.26 | −1.08 |
| 8/ 2 | 0 | 110 | 4.07 | 4.29 | −0.22 | 5.18 | 5.00 | 0.18 |
| 8/ 3 | 0 | 100 | 4.07 | 4.00 | 0.07 | 5.18 | 4.93 | 0.25 |
| 8/ 4 | 9 | 80 | 4.07 | 5.27 | −1.20 | 5.18 | 4.53 | 0.65 |
| 8/ 5 | 9 | 90 | 4.07 | 6.51 | −2.44 | 5.18 | 7.82 | −2.64 |
| 8/ 6 | 9 | 70 | 4.07 | 7.33 | −3.26 | 5.18 | 12.67 | −7.49 |
| 8/ 7 | 9 | 175 | 4.07 | 6.62 | −2.45 | 5.18 | 4.35 | 0.83 |
| 8/ 8 | 9 | 100 | 4.07 | 5.63 | −1.56 | 5.18 | 7.25 | −2.07 |
| 8/ 9 | 9 | 100 | 4.07 | 6.02 | −1.95 | 5.18 | 6.39 | −1.21 |
| 8/10 | 9 | 135 | 4.07 | 4.18 | −0.11 | 5.18 | 4.31 | 0.87 |
| 8/11 | 9 | 180 | 4.07 | 3.05 | 1.02 | 5.18 | 4.73 | 0.45 |
| 8/12 | 9 | 135 | 4.07 | 2.66 | 1.41 | 5.18 | 3.62 | 1.56 |
| 8/13 | 3 | 110 | 4.07 | 2.32 | 1.75 | 5.18 | 1.61 | 3.57 |
| 8/14 | 0 | 150 | 4.07 | specimen lost | | 5.18 | — | — |
| 8/15 | 0 | 140 | 2.86 | 2.21 | 0.65 | 1.14 | 3.35 | −2.21 |
| 8/16 | 0 | 95 | 4.07 | 3.22 | 0.85 | 5.18 | 1.24 | 3.94 |
| 8/17 | 0 | 140 | 4.07 | 4.03 | 0.04 | 5.18 | — | — |
| 8/18 | 0 | 120 | 4.07 | 2.60 | 1.47 | 5.18 | 4.71 | 0.47 |
| 8/19 | 0 | 110 | 4.07 | 2.43 | 1.64 | 5.18 | — | — |
| 8/20 | 0 | 135 | 4.07 | 4.04 | 0.03 | 5.18 | — | — |
| 8/21 | 0 | 115 | 4.07 | 3.68 | 0.39 | 5.18 | 5.45 | −0.27 |
| 8/22 | 0 | 120 | 4.07 | 4.10 | −0.03 | 5.18 | 5.12 | 0.06 |

tinued, and the polyuria returns promptly upon cessation of the treatment. Normal cats, on the other hand, show either a slight decrease in urine volume or none at all, and even that small response ceases after the second or third day, whether pitressin injections are continued or not (table 2).

These results are in line with the report of Smith and MacKay (19) that d.i. patients commonly do not show the marked increase in sodium and chloride excretion following posterior pituitary extract injection which they observed in normal subjects. They considered the difference in effect of the extract on salt balance in normal and d.i. subjects as an accidental result of the difference in effect on urine volume; we are rather inclined to the view that our results indicate that the effects of pitressin on salt and water excretion are two independent mechanisms. It is generally accepted that posterior lobe function is very important in the regulation of water exchange in the intact organism; it is not so evident that a normally functioning posterior lobe is essential for health so far as salt balance is concerned. D.i. animals and patients remain in good health and good salt balance on a salt intake not differing from that in

TABLE 3. SALT EXCHANGE DURING PITRESSIN INJECTIONS IN NON-ADRENALECTOMIZED CATS, M.EQ. DAILY

| Diabetes insipidus cats | | | | | | | | Control cats | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cat number | Days injected | Cl exchange | | | Na exchange | | | Cat number | Days injected | Cl exchange | | | Na exchange | | |
| | | Av. intake | Av. output | Av. balance | Av. intake | Av. output | Av. balance | | | Av. intake | Av. output | Av. balance | Av. intake | Av. output | Av. balance |
| 1 | 3 | 4.07 | 3.67 | 0.40 | 5.18 | 5.58 | −0.40 | 9 | 4 | 4.07 | 10.81 | −6.74 | 5.18 | 13.41 | −8.23 |
| 2 | 3 | 4.07 | 5.90 | −1.83 | 5.18 | 4.75 | 0.43 | 10 | 4 | 4.07 | 6.89 | −2.82 | 5.18 | 8.43 | −3.25 |
| 3 | 4 | 4.07 | 5.21 | −1.14 | 5.18 | 8.65 | −3.47 | 11 | 4 | 4.07 | 10.28 | −6.21 | 5.18 | 11.39 | −6.21 |
| 18 | 4 | 4.07 | 5.73 | −1.66 | — | — | — | 12 | 3 | 4.07 | 10.07 | −6.00 | 5.18 | 7.07 | −1.89 |
| average | | 4.07 | 5.13 | −1.06 | 5.18 | 6.33 | −1.15 | average | | 4.07 | 9.51 | −5.44 | 5.18 | 10.08 | −4.90 |

the normal diet, but the water requirement of such individuals is much higher than normal. White and Findley (23) have reported, however, a slight delay in excretion of large doses of ingested NaCl in d.i. patients as compared with normal subjects.

*Sodium and chloride exchange after adrenalectomy in pitressin-injected and non-injected control and d.i. cats.* D.i. cats deprived of their adrenals do not show the decrease in serum sodium and chloride concentration usually observed after adrenalectomy only in non-d.i. cats. If, as has been suggested (3–5), changes in serum electrolytes in adrenal insufficiency are mainly reflections of changes in excretion, it might be expected that d.i. cats would not show the usual negative post-adrenalectomy sodium

TABLE 4. SALT EXCHANGE AFTER ADRENALECTOMY

| | Time after adrenalectomy | Cl exchange | | | | Na exchange | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Intake | Output | Balance | Cumulative post-adrex. balance | intake | Output | Balance | Cumulative post-adrex. balance |
| | | *m. eq.* | *m. eq.* | *m. eq.* | *m. eq.* | *m.eq.* | *m.eq.* | *m.eq.* | *m.eq.* |
| Control cats | 1st day | 1.86 | 4.03 | −2.17 | − 2.17 | 3.11 | 6.89 | −3.78 | − 3.78 |
| | 2nd day | 2.02 | 5.12 | −3.10 | − 5.27 | 3.32 | 9.51 | −6.19 | − 9.97 |
| | 3rd day | 1.69 | 4.78 | −3.09 | − 8.36 | 2.24 | 4.98 | −2.74 | −12.71 |
| | Entire post-adrenalectomy period | 12.02 | 27.12 | (3 cats) | −15.10 | 14.68 | 36.43 | (3 cats) | −21.75 |
| Diabetes insipidus cats | 1st day | 3.54 | 6.29 | −2.75 | − 2.75 | 3.58 | 8.87 | −5.29 | − 5.29 |
| | 2nd day | 1.41 | 4.33 | −2.92 | − 5.67 | 1.44 | 5.49 | −4.05 | − 9.34 |
| | 3rd day | 1.64 | 3.05 | −1.41 | − 7.08 | 0.79 | 1.06 | −0.27 | − 9.88 |
| | Entire post-adrenalectomy period | 7.84 | 15.01 | (5 cats) | − 7.17 | 7.47 | 18.46 | (4 cats) | −10.99 |
| Control cats with pitressin | 1st day | 5.93 | 8.48 | −2.55 | − 2.55 | 3.71 | 8.63 | −4.92 | − 4.92 |
| | 2nd day | 2.42 | 7.11 | −3.69 | − 6.24 | 4.90 | 11.95 | −7.05 | −11.97 |
| | 3rd day | 2.70 | 5.42 | −2.72 | − 8.96 | 3.44 | 9.08 | −5.64 | −17.61 |
| | Entire post-adrenalectomy period | 17.64 | 31.01 | (6 cats) | −13.37 | 15.58 | 35.16 | (3 cats) | −19.58 |
| Diabetes insipidus cats with pitressin | 1st day | 4.40 | 6.43 | −2.03 | − 2.03 | 2.83 | 7.41 | −4.58 | − 4.58 |
| | 2nd day | 3.05 | 5.60 | −2.55 | − 4.58 | 3.26 | 7.36 | −4.10 | − 8.68 |
| | 3rd day | 2.76 | 3.81 | −1.05 | − 5.63 | 3.28 | 5.83 | −2.55 | −11.23 |
| | Entire post-adrenalectomy period | 15.38 | 28.63 | (7 cats) | −13.25 | 11.89 | 27.30 | (3 cats) | −15.41 |

and chloride balances. This, however, is not the case (table 4). The post-adrenalectomy survival time of the d.i. cats is much shorter than that of the non-polyuric animals; by the end of the second day, they almost invariably show severe symptoms of adrenal insufficiency, and seldom survive beyond the third day. During the time that they remain in a state of health approaching that of the controls, however, they show a negative sodium and chloride daily balance of the same order.

Although the total loss of electrolytes for the entire post-adrenalectomy period is less in the d.i. animals than in the non-polyuric, this appears to be merely a reflection of the shorter survival time. By the end of the third post-adrenalectomy day, the d.i. cats have lost by excretion about the same amount of sodium and chloride as the controls; the small differences which appear in the table are within the range of individual variation. Both groups lose sodium more rapidly than chloride. The only notable

difference between the two groups is that the d.i. cats apparently reach their own particular peak rate of daily excretion earlier in the period of insufficiency than do the controls. This is in accord with the more rapid development of symptoms in the former.

Three days after adrenal removal, the control cats are in early insufficiency; the d.i. cats are moribund or dead. The amounts of sodium and chloride excreted by the 2 groups during this period are approximately the same, and yet the behavior of the serum electrolytes is quite different. In the d.i. cats, the excess excretion of these electrolytes is not accompanied by changes in their level in the serum, while in the controls serum chloride by the third post-adrenalectomy day has already dropped from an average of 115 m.eq. per liter to about 105, and serum sodium from 145 to 130. In other words, by far the greater part of the reduction in serum sodium and chloride concentration observed in adrenalectomized non-polyuric cats takes place within a period of time when the d.i. cats show no change in such concentration at all. Striking reduction in serum sodium and chloride in the early part of adrenal insufficiency has also been shown by Loeb et al. (4), Harrop and Weinstein (24) and Zwemer and Sullivan (25).

The post-adrenalectomy negative sodium and chloride balances are not affected appreciably by pitressin injections. This was equally true whether the animals were prepared by a course of injections before the final adrenal operation, or whether injections were started at the time of the operation; therefore, no distinction is made in table 4 between the two methods of treatment. The urinary salt concentration and the daily output of salt were about the same during pitressin injections before adrenalectomy as they were during adrenal insufficiency without pitressin, but if the two conditions were combined there was no additive effect. The total salt output for the entire post-adrenalectomy period was greater in the d.i. cats given pitressin than in the non-injected d.i. animals, but this appears to be merely a consequence of the life-prolonging effect of pitressin on adrenalectomized d.i. cats (2), for it does not significantly alter the salt exchange during the early part of insufficiency.

## DISCUSSION

Some of these results offer a possible explanation for the observation that serum sodium chloride declines in concentration after adrenalectomy in non-d.i. cats, but not in d.i. cats. This can best be made clear by presenting a simple calculation. If one accepts as a reasonable assumption that the fluid in the cat's body available for the solution of chloride is about 30% of the body weight (26), a 2.5 kg. cat would have about 750 cc. of such fluid. The data in table 4 show that the average total negative chloride balance for the first three post-adrenalectomy days in all cats not receiving pitressin is 7.56 m.eq. Since this portion of the excreted chloride did not come from the food, we may regard it as derived from the chloride-containing fluids of the body. It is evident that if this loss of chlorides is accompanied by an equivalent loss of water, the concentration in the fluid left behind would be unchanged, but if the chloride loss exceeds the water loss, the chloride concentration in the body fluids might be expected to be reduced. The amount of the reduction in chloride concentration in the chloride-containing fluids of a cat that had not yet reached the stage of negative water balance, would be 7.56:750, or about 10 m.eq. per liter. This theoretical figure exactly corresponds with the actual experimental findings for non-d.i. cats, as shown in the preceding section of this paper.

We have already pointed out (2) that water loss is much more prompt after adrenalectomy in d.i. cats than in non-d.i. animals; indeed, in the 3-day average survival time of the d.i. cats, the total negative water balance is as great as that for the

entire survival time, averaging about 8 days, of the non-polyuric controls. Non-d.i. cats, in fact, commonly remain in good health for 3 or 4 days after adrenal removal, and often do not begin to show a negative water balance during this early part of the insufficiency period. Since both groups of animals lose chlorides at about the same rate following adrenalectomy, and the d.i. animals lose water much more promptly, it will be seen that conditions are favorable for a more pronounced drop in chloride concentration in body fluids of the non-d.i. cats. If this line of reasoning is correct, it might be expected that the d.i. animals would show blood volume changes in their 3-day survival period of about the same magnitude as those shown by non-d.i. animals in their longer survival period; our data on this point are not complete, but red blood cell counts and estimates of total blood solids (unpublished data) made on d.i. and non-d.i. cats before and after adrenalectomy indicate that about the same degree of hemoconcentration occurs in both during adrenal insufficiency.

On the basis of this argument, we can conclude that the explanation for the failure of serum chloride to show a decline in concentration during adrenal insufficiency in d.i. cats lies in the relatively unstable water balance conditions in such animals. D.i. animals may be regarded as always in a state of potential negative water balance, which may with greater ease than in the normal animal be thrown into an actual negative state. The suppression of thirst in d.i. animals after adrenalectomy is not accompanied by an equivalent decrease in urine volume, although the polyuria does decline somewhat; this has been more fully discussed in a previous paper (27). We may summarize the argument by saying that in the d.i. cats, unlike the controls, the excess excretion of sodium and chloride after adrenalectomy is not accompanied by changes in serum concentration of these electrolytes because in the former, the negative NaCl balance immediately following adrenal removal is accompanied by a strongly negative water balance, so that the concentration of NaCl in the body fluids is relatively unaffected, whereas in the non-polyuric animals, the salt balance becomes strongly negative early in the period of corticoadrenal insufficiency at a time when there is still a fairly good water balance, so the net result is a lowering of salt concentration in the body fluids.

Certain results which we previously reported (2) on the behavior of serum electrolytes after adrenal removal in d.i. cats seemed to offer definite evidence that the normally innervated posterior lobe participates in certain phases of the metabolism of sodium and chloride. Considered in the light of the present findings, however, it seems more likely that such participation, as it is manifested after adrenalectomy at least, is an indirect one, and a consequence of the effect of the posterior lobe on water metabolism. There remains, however, other evidence that under certain conditions the posterior lobe may be more directly concerned with salt metabolism. This evidence includes the observations on the effect of posterior lobe extract on salt excretion (table 2 and 3). It has been postulated that posterior lobe extract mobilizes salt from the tissues (13, 20), and if the excretion of this mobilized salt is prevented, as by nephrectomy, there is a marked increase in blood chloride (14, 28); the validity of this theory is questionable, however, in view of the results of McIntyre and Van Dyke (29). At any rate, evidence as to the function of a gland, based solely on effects of extract injections, is indirect, and conclusions based on such evidence must await final proof that the injections constitute an actual duplication of the functions of the living gland.

More direct evidence of the importance of the posterior lobe for normal salt metabolism is found in the reports that there is a deficient ability to concentrate salt in the urine during d.i. (30, 31), and a delay in the excretion of ingested salt by d.i. patients (23).

Unna and Walterskirchen (20) regarded the effect of pituitary extract on urine volume as secondary to the mobilization of salt, for they found that if dogs are primed with large doses of ingested salt, pituitary injection led not only to an increased salt excretion, but increased water excretion as well. Their results, however, may be explained on the basis that their dogs on extract were excreting salt at or near the concentration ceiling, and any increase in salt excretion would necessarily result in an augmented urine volume. In our experiments, it is quite apparent that the effect of pitressin on urine output cannot be secondary to the salt effect, for in the normal cats water excretion was relatively little affected by the injections but salt excretion markedly increased, while in the d.i. cats water excretion was greatly reduced and salt excretion affected very little. It is difficult to understand why this should be the case, but it was found fairly consistently, and Smith and MacKay (19) noted the same phenomenon in a d.i. patient. One possible explanation is that the extract used (Parke, Davis, pitressin) may not contain all posterior lobe principles that are concerned in salt excretion, and in the normal animals the missing principle or principles were already present in the animal, supplied by his own gland. This possibility is strengthened by the observation in two d.i. cats (not shown in the tables) that subcutaneous implants of whole dried posterior lobe substance led temporarily to fully as great a negative salt balance as was observed in any normal cat receiving injections.

## SUMMARY

When the supraoptico-hypophyseal tracts are interrupted in a cat, there is usually a very sudden increase in urine volume at the onset of the transient polyuria, and again at the onset of the permanent polyuria. Accompanying each sudden urine increase is an equally abrupt increase in chloride excretion which is later compensated for, so that in the long run there is as good balance between chloride intake and output in the diabetes insipidus animals as in normals. Although these sudden changes in chloride output appear coincidentally with apparent changes in the functional capacity of the posterior lobe, evidence is presented that the unusually large chloride output is not a direct effect of a sudden alteration in posterior lobe function, but rather an incidental effect of the sharp increase in urine volume.

Injection of three pressor units of pitressin three times daily leads to a strongly negative sodium and chloride balance, but the effect is temporary and is followed by a compensatory retention, whether the injections are continued or not. Salt excretion is much less affected by pitressin in diabetes insipidus animals than in normals, although water excretion is more markedly affected in the former; it is concluded that the effects of pitressin on urine volume and on salt excretion are two independent mechanisms.

After adrenalectomy, diabetes insipidus cats show negative sodium and chloride balances the same as non-polyuric animals for the first few days of adrenal insufficiency, although the total post-adrenalectomy loss of these electrolytes is not as great in the d.i. animals because their survival time is much shorter. In the d.i. cats, unlike the controls, the excess excretion of electrolytes is not accompanied by changes in sodium and chloride concentration in the serum. This is thought to be due to the fact that in the d.i. cats, the negative chloride balance immediately following adrenal removal is accompanied by a strongly negative water balance, so that the concentration of chloride in the body fluids is unaffected, whereas in the non-polyuric animals, the salt balance becomes strongly negative early in adrenal insufficiency at a time when there is still a fairly good water balance, so the net result is a lowering of salt concentration in the body fluids.

Although pitressin injections in a normal animal lead to a negative salt balance

about equal to that found after adrenalectomy, when the two conditions are combined there is no additive effect; that is, pitressin injection after adrenal removal does not make the sodium and chloride balances any more strongly negative than they are without such injections.

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# VITAMIN B COMPLEX AND ADRENALECTOMY[1]

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CONSIDERABLE INTEREST has been recently shown in the possible relationships between the vitamin requirements of adrenalectomized animals and the necessity of the adrenal cortex for the utilization of some of the members of the vitamin B complex.

Most of this interest has been stimulated by the work of Verzár and Laszt, which has been largely summarized in Verzár's recent book (1). Verzár and his coworkers have repeatedly emphasized their theory that adrenal insufficiency is in reality a secondary avitaminosis, with particular reference to the necessity of the adrenal cortex for the phosphorylation and subsequent incorporation of riboflavin, thiamin, and nicotinic acid into enzyme-coenzyme systems (2, 1).

The present paper purports to reexamine this question with respect to vitamin therapy, and to present results of various therapies in adrenalectomized rats with thiamin, cocarboxylase, riboflavin, riboflavin phosphate ester, nicotinic acid, pyridoxine (vitamin $B_6$), pantothenic acid, Factor W, various vitamin concentrates, and riboflavin and its phosphate ester in adrenalectomized guinea pigs. Each substance will be separately discussed.

## 1. *Thiamin and Cocarboxylase Therapy in Adrenalectomized Rats*

Laszt (3) maintained that thiamin deficient, adrenalectomized rats develop a marked bradycardia, which could be cured by thiamin only if adrenal cortical extract was administered, and that cortin without thiamin was likewise ineffective. Blood pyruvate was increased in adrenalectomized normal and thiamin-deficient rats, and could not be decreased by cortin or thiamin separately, but could if they were given together. Laszt (3) and Verzár (1) postulated from this indirect evidence, that thiamin cannot be phosphorylated in vivo to cocoarboxylase in the absence of the adrenal cortex.

*Experiment 1. Parenteral and oral cocarboxylase* (6/24/39). Rats of mixed sexes of the Sprague-Dawley strain of albino rats,[2] 4 to 6 weeks old, were divided into 2 groups of 10 rats each, average body weight 100 gm. (range, 91–113). Each group was caged in one cage, and fed Rockland 'complete rat ration';[3] 1% NaCl, 0.2% $NaHCO_3$ drinking fluid (hereafter called salt drinking fluid) was offered for 24 hours following adrenalectomy. *Group 1*, serving as controls, received 0.5 cc. 0.9% NaCl subcutaneously daily for 3 days after removal from the salt drinking fluid, then intraperitoneally for 3 days, thereafter orally in sweetened water from a needleless tuberculin syringe.

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[2] Sprague-Dawley, Inc., Madison, Wis.

[3] Arcady Farms Milling Co., Chicago, Ill. Riboflavin ca. 3.8, and thiamin, ca. 1.2 γ per gm.; Na, 1.4%, and K, 0.76%.

*Group 2* received the same amounts of saline in the same way, but each 0.5 cc. contained 1000 γ of crystalline cocarboxylase.[4] *Group 1* survived 10.6 days (range 7–14) while *group 2* survived 9.0 days (5–12). The slopes of the daily weight-loss curves were comparable.

*Experiment 3. Oral thiamin and cocarboxylase* (6/1/40). Sprague-Dawley female rats approximately 2.0 to 2.5 months of age, were divided into 2 groups of 3 rats each, average body weight 141 (131–157) gm., each group being caged together and fed the Rockland rat food. *Group 1* received 200 γ of crystalline thiamin hydrochloride per gm. of food, and *group 2* the same amount of cocarboxylase in the food. The daily food consumption on successive days, together with the calculated vitamin intake (noninclusive of the vitamin content of the Rockland diet. For this content, cf. footnote above on Rockland 'complete rat ration'), per rat per day on successive days, are seen in table 1.

TABLE 1. EFFECT OF THIAMIN AND COCARBOXYLASE ON APPETITE OF ADRENALECTOMIZED RATS

Gm. of food intake per rat per day on successive days from time of removal of salt drinking fluid

| Days | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Grp. 1* (thiamin) | 3.0 | 3.7 | — | 5.3 | 3.6 | 3.3 | 4.6 | 4.0 | — | 4.5 | — |
| *Grp. 2* (cocarbox.) | 6.0 | 4.0 | 5.4 | 5.0 | 3.0 | 5.0 | 5.4 | 4.5 | 4.3 | 5.3 | 5.0 |

Calculated γ of thiamin and cocarboxylase intake per rat per day on successive days, from time of removal of salt drinking fluid

| Days | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Grp. 1* (thiamin) | 600 | 740 | — | 1060 | 720 | 660 | 920 | 800 | — | 900 | — |
| *Grp. 2* (cocarbox.) | 1200 | 800 | 1080 | 1000 | 600 | 1000 | 1080 | 900 | 860 | 1060 | 1000 |

*Group 1* survived an average of 10 days, and *group 2*, 11.5 days. No difference in the slopes of the daily weight-loss curves were seen. Thus even though *group 2* consumed slightly more food than *group 1*, no beneficial effects of the contained cocarboxylase as compared with thiamin resulted.

A third experiment involving 24 rats, in which 1000 γ of thiamin hydrochloride and an equivalent amount of cocarboxylase (ca. 1500 γ) were subcutaneously injected daily, revealed no difference in appetite, daily weight-loss, and survival.

It is obvious from these results that relatively massive, yet non-toxic[5] doses of thiamin hydrochloride and its pyrophosphate, cocarboxylase, had no beneficial effects on adrenalectomized rats, nor was there a difference in action of the two substances. This result is in direct accord with the observations of Ochoa and Rossiter (6) who found no decrease in measured cocarboxylase in in extremis adrenalectomized rats, and fails to support the phosphorylation hypothesis of Verzár and Laszt (1, 3).

### 2. *Riboflavin and Riboflavin Phosphate Ester Therapy in Adrenalectomized Rats*

Verzár and Laszt (7), Verzár, Hübner, and Laszt (8), Verzár and Laszt (9), and Verzár (1), postulated that one of the major functions of the adrenal cortex is to phosphorylate riboflavin. According to this theory, riboflavin is not available for incorporation into flavin coenzymes unless the adrenal cortex is present, and the adrenalectomized animal dies of a

[4] All pure vitamins used in this study were supplied in generous amounts by Merck & Co., Inc., Rahway, N. J., unless otherwise specified in the text.

[5] Molitor and Sampson (4) report a minimal lethal dose of 250 mg. thiamin intravenously in rats; while Sure (5) found disturbances in reproduction and lactation in rats receiving 100–400γ orally daily.

'secondary avitaminosis $B_2$' just as discussed above in the example of thiamin and cocarboxylase. It is claimed that the adrenalectomized animal will survive if riboflavin phosphate ester is administered.

Jiminez-Diaz (10), working with kidney slices, reported a decreased activity of amino oxidase after adrenalectomy; and thus possibly supporting Verzár and Laszt in that the flavin coenzyme component (of amino oxidase) decreases in adrenalectomy.

Russell and Wilhelmi (11) confirmed and extended the work of Jiminez-Diaz, finding that oxidative deamination and oxidation of keto-acids both decrease in adrenalectomized rat kidney slices. Wilhelmi (12) also found that cortin restores the rates to normal in the case of the ketoacids, and to supernormal levels in that of the amino acids. Cortin also raised the oxidation and deamination to supernormal levels in normal rats. In this connection, Klein (13) succeeded in obtaining supernormal levels of d-amino oxidase activity in rat liver by thyroid administration. He indicated that the increase was due to an increase in the concentration of the protein component of the oxidase rather than the flavin component.

Axelrod, Sober, and Elvehjem (14, 15) reported a d-amino oxidase decrease in riboflavin deficiency. Ochoa and Rossiter (16), however, directly determined the flavin-adenine-dinucleotide content of normal and in extremis adrenal insufficient rat brain, heart, liver, and kidney, reporting that although there was a decrease of the flavin component in riboflavin deficiency which was restored to normal by administration of riboflavin, there was no difference in the flavin component content of tissues of normal and adrenalectomized rats.

The collected evidence presented above thus strongly contraindicates that the adrenal cortex influences the flavin component of any oxidases in tissue respiration, but that it probably does affect the protein components of the oxidases. All this is, however, directly opposed to the Verzár and Laszt thesis that flavin phosphorylation has any relation to the adrenal cortex.

An attempt to confirm Verzár and Laszt that riboflavin phosphate ester will prolong life in adrenalectomized rats was made by Pijoan and Oberg (17), who claimed positive results. Pijoan (18) stated that the cessation of ester therapy after 3 weeks of treatment had no detrimental effect on his rats, and that, in effect, the ester supported life long enough to allow compensatory hypertrophied adrenal bodies to indefinitely maintain the animals. It was also stated that Verzár privately confirmed this finding. Pijoan communicated that there was no effect of the ester on survival of adrenalectomized cats and dogs, or in Addison's disease.

Pijoan and Oberg (17) used a flavin ester prepared by the method of Karrer, Saloman, Schopp, and Benz (19), of Kuhn and Rudy (20), and by applying Kuhn's method to yeast which Stern (21) used to extract photoflavins from liver. The rats were fed the flavin-free diet of Verzár and Laszt (7, 1). No dosages other than volumes of solutions of the ester were given, nor is there a statement that the ester preparations were chemically satisfactory.

A similar criticism can be made of Verzár's flavin ester studies in relation to the adrenal cortex. This author could find no publications of the Verzár school in which pure flavin ester was used. Concentrates of liver and yeast were in every case employed. There are, however, some experiments on the in vitro phosphorylation of riboflavin, in which it was claimed that adrenalectomized rat tissue phosphorylated riboflavin to a less extent than normal tissue, and that less bound flavin is found in adrenalectomized rat, dog and cat liver (8).

In connection with Verzár and Laszt' studies of prolonged survival of adrenalectomized rats with yeast and liver concentrates (rich in bound flavin), Marrazzi and Gaunt (22) fed adrenalectomized rats different levels of yeast and found no effect on survival. As a matter of fact, Bruce and Wien (23) found that 5% baker's yeast added to their stock diet (containing about the same level of yeast as that used by Marrazzi and Gaunt) actually shortened survival of adrenalectomized rats, presumably because of increased potassium in the diet.

In addition to the above objections, Verzár and Laszt (7), Verzár (1), and Pijoan and Oberg (17) always used a flavin-free diet in their studies, which consisted of casein, starch, fat, salts, cod liver oil, and riboflavin or thiamin as supplements, depending on the type of study. Verzár states (1, p. 20) that adrenalectomized rats fed this B-poor diet died within 13 days, but that under no circumstance could cereal grains be fed, or a great number of the animals would survive indefinitely. The work of Pico (24), quoted by Laszt (3) demonstrated a marked sensitivity of adrenalectomized rats to avitaminosis B, while Csik and Méhes

(25) found that survival of adrenalectomized rats markedly decreased after vitamin B complex deficiency. This observation is also borne out by the experiments of Bruce and Wien (23) who demonstrated an increased sensitivity of riboflavin-deficient rats to adrenalectomy. It is important to note that Axelrod, Sober, and Elvehjem (14, 15) have shown that a deficiency in d-amino oxidase (which contains phosphorylated riboflavin) brought about by riboflavin deficiency, is under certain conditions restored to the normal level by riboflavin only if other members of the vitamin B complex (e.g. Factor W) are simultaneously administered. In addition to these facts, it is well known that any condition of malnutrition, infection, stress, etc,. promotes collapse and early death in adrenalectomized animals. The fact that Verzár and Laszt, and Pijoan and Oberg used a diet deficient in all the members of the vitamin B complex except thiamin and riboflavin indicates that they were dealing with a weakened animal. Moreover, that Verzár and Laszt (1) found that 'Körnerfutter' led to indefinite survival in the majority of their adrenalectomized rats, demonstrates that their rats developed hypertrophied adrenals readily, which maintained the animals if avitaminosis B in general was avoided.

Weslaw and Wroblewski (26) administered a daily dose of 120–240 γ of I. G. Farbenindustrie (Bayer) riboflavin phosphate ester to adrenalectomized rats, but found no beneficial effects. Nelson (27), using a commercial synthetic flavin ester, also reported negative results when 30 to 120 γ was administered daily.

In a recent publication, which appeared while this manuscript was in preparation, Bruce and Wien (23) made a fairly critical examination of the Verzár and Laszt avitaminosis $B_2$ theory. They found that rats lacking both riboflavin and cortical hormone died unless both substances were administered. Riboflavin prolonged survival of riboflavin-deficient adrenalectomized rats, but neither substance could support life without the other. Oral and parenteral daily administration of 25 to 40 γ of Bayer 'Lactoflavinphosphorsäure' (see also Weslaw and Wroblewski, loc. cit.) to normal and riboflavin deficient adrenalectomized rats had no effect different from that of riboflavin.

During the course of the present study, an analysis of a commercial riboflavin phosphate ester preparation was made, and was found to be very low in phosphorus. The analyses were as follows.

| *Formula* | *Calculated* | | | *Found* | | |
|---|---|---|---|---|---|---|
| | *C* | *H* | *P* | *C* | *H* | *P* |
| $C_{17}H_{21}O_9N_4P$ | 44.8 | 4.4 | 6.8 | | | |
| $C_{17}H_{20}O_9N_4PNa$ | 42.7 | 4.2 | 6.5 | 41.73 | 5.08 | 1.75 |
| $C_{17}H_{19}O_9N_4PNa_2$ | 40.8 | 3.8 | 6.2 | | | |

This product was synthesized by the method of Kuhn and Rudy (20), which was also the synthesis employed in the preparation used by Pijoan and Oberg, Weslaw and Wroblewski, Nelson, and Bruce and Wien, although Pijoan and Oberg also used another method (see above). Because of this unreliability, a variety of different methods of synthesis was carried out in these laboratories, which will be reported elsewhere. This study showed that no two syntheses performed by the Kuhn and Rudy procedure yielded a similar product, necessitating careful elementary analyses on all yields obtained before the product could be deemed satisfactory for experimental use. Moreover, other methods of the synthesis were found to be remarkably difficult and variable, likewise necessitating elementary analyses. In the experiments reported below, the reliability of the product was confirmed in this way before it was administered.

The following experiments purport to examine the effects of relatively massive doses of riboflavin and riboflavin phosphate ester on adrenalectomized rats and guinea pigs.

Three experiments, involving a total of 48 rats, in which riboflavin was administered orally and parenterally in doses graded from 400 to 1000 γ, daily, revealed the

same negative results, that riboflavin in relatively massive, yet non-toxic[6] oral and parenteral doses exert no beneficial effect on weight maintenance, food intake, or survival of adrenalectomized rats of various ages and both sexes when they are fed a diet adequate for normal rats.

*Experiment 6. Parenteral riboflavin and riboflavin phosphate ester* (4/23/40). Sprague-Dawley male rats were divided into 3 groups of 10 rats each, average body weight, 222 (198–259) gm. Each group was caged together and fed Rockland rat diet. Salt drinking fluid was offered for 48 hr. after adrenalectomy. *Group 1*, the controls, received 0.5 cc. peanut oil subcutaneously on the 2nd day after the operation, 0.25 cc. on the 3rd and 4th days, 1 cc. saline thereafter for 4 days, and 0.5 cc. saline for the next 10 days. *Group 2* received riboflavin suspended in the same volumes of the same solvents on the same days. The amounts of riboflavin were 1000 and 500 $\gamma$ in the 0.5 and 0.25 cc. of peanut oil, respectively (protracted absorption?), 2000 $\gamma$ in the 1 cc. of saline, and 1000 $\gamma$ in the final 0.5 cc. of saline. *Group 3* received sodium riboflavin phosphate ester in a similar way, in comparable doses on the same days. This ester preparation was prepared in these laboratories by the Kuhn and Rudy synthesis (20), and analyzed: C, 37.01; H, 4.16; P, 6.4%; high in true ester. *Group 1*, the controls, survived 24 (12, 13, 15, 15, 19, 27, 30, 35, 36, 37) days. *Group 2* survived 20 (2*, 6*, 10, 14, 15, 16, 21, 22, 24, 38) days, the asterisks signifying that these animals developed 'sniffles'[7] and hence were not included in the average. *Group 3*, the ester treated rats, survived 22 (15, 16, 17, 19, 21, 21, 21, 25, 27, 37) days. The daily weight loss curves and average daily food consumption per rat per day of the 3 groups were fairly similar.

*Experiment 8. Parenteral riboflavin and riboflavin phosphate ester* (8/28/39). Female rats of the 'La Jolla' strain (31) were divided into 2 groups of 9 and 7 rats each, average body weight 210 (152–275) gm. Each group was caged together and fed a mixture of Crown growing mash (Maney Bros. Mill and Elevator Co., Minneapolis, Minn.) and Champion mink food (Champion Animal Food Co., Minneapolis), which have given growth curves comparable to those obtained with Purina Dog Chow and Rockland rat food. No salt drinking fluid was offered following the operation. *Group 1* received 100 $\gamma$ of riboflavin per 100 gm. body weight suspended in 0.25 cc. $H_2O$ subcutaneously on the 1st day after the operation, and 25 $\gamma$ per 100 gm. body weight in 0.5 cc. $H_2O$ thereafter daily for 10 days. On the 11th day, 200 $\gamma$ per 100 gm. body weight was administered. *Group 2* received sodium riboflavin phosphate ester in the same amount on the same days. The ester was kindly supplied by the Winthrop Chemical Company, and was an I. G. Farbenindustrie product, prepared by the Kuhn and Rudy synthesis (20) (see discussion and analysis of a commercial preparation, above). *Group 1* survived 13 (10, 11, 11, 11, 13, 14, 14, 16, 16) days, and *group 2* 14 (10, 11, 13, 13, 13, 16, 25) days. The daily weight loss curves and food consumptions were similar.

*Experiment 9. Parenteral riboflavin and riboflavin phosphate ester in adrenalectomized guinea pigs* (7/16/39). Six male guinea pigs were adrenalectomized by a technic somewhat similar to that described by Schachter and Bebee, Jr. (32), and which is discussed elsewhere (33). *Group 1*, the controls, consisted of 3 guinea pigs, average body weight 472 gm. *Group 2* consisted of 3 animals, average body weight 474 gm. The animals were separately caged and fed a diet of alfalfa hay, fresh cabbage daily, and whole oats. Tap water was offered. The animals of *group 1* received 250 $\gamma$ of ribo-

[6] Kuhn and Boulanger (29) found that 340 mg. riboflavin orally administered, caused no injurious effects in mice. Cf. also Widenbauer and Wedemeyer (30).

[7] This respiratory infection was very seldom encountered in this work, as the experiments were carried out in a thermostatically controlled, air-conditioned room at 26° C., with no draughts. The cages were sheltered in deep shelves.

flavin suspended in 0.5 cc. $H_2O$ subcutaneously on the day of removal of the 2nd adrenal, and thereafter daily until death occurred. *Group 2* received similar amounts of sodium riboflavin phosphate ester (the same preparation that was used in *experiment 8*) on the same days.

All animals decreased rapidly in weight, *group 1* surviving 3.9 (3.7, 6.0, 2.0) days, and *group 2*, 4.7 (4.5, 5.5, 4.0) days. This can be compared with 4.6 days average survival of 20 adrenalectomized guinea pigs described elsewhere (33). The weight loss curves were similar.

A total of 8 experiments were performed on possible effects of riboflavin and its phosphate ester on adrenalectomized rats, of which *experiments 6* and *8*, described above, were typical. These studies involved 150 rats of various ages and sexes, and employed both oral and parenteral routes in administering the two substances, in doses graded from 100 to 2000 γ daily. The results of these 8 experiments, and those of *experiment 9*, on guinea pigs, clearly showed that neither riboflavin or its phosphate ester administered orally and parenterally in amounts in great excess of dietary requirement, have any beneficial effects on weight maintenance, appetite for various adequate diets, or survival of adrenalectomized rats of both sexes and various ages, or of survival of adrenalectomized guinea pigs; nor was there a difference in action of the vitamin and its phosphorylated form in these animals. This result supports and extends the preponderantly negative evidence presented in the discussion in this section, that the adrenal cortex has any influence on the phosphorylation of riboflavin as far as life maintenance is concerned.

### 3. *Nicotinic Acid Therapy in Adrenalectomized Rats*

Verzár (1) postulated a relationship between pellagra and the adrenal cortex, correctly pointing out, however, that rats do not require nicotinic acid nor do they develop pellagra (cf. also 34, 35). There are, however, isolated observations that nicotinic acid may be of benefit in avitaminosis B in rats (36). Von Euler, Schlenk, Melzer and Högberg (37) showed that tissues of avitaminosis B rats contain less nicotinamide and cozymase than normal rats, and that feeding the former increased both. Axelrod, Madden and Elvehjem (38), in a study of cozymase in nicotinic acid deficiency in the dog and pig, incidentally found that excessive administration of nicotinic acid had no effect on the cozymase content of rats. Barány (39) was interested in pursuing the Laszt and Verzár idea that the adrenal cortex is essential for various vitamin phosphorylations, and examined the possible influence of adrenalectomy on the cozymase content of rat tissues. He found that in extremis adrenal insufficient rats suffered no change from normal of tissue cozymase (containing phosphorylated nicotinamide).

Although the rat probably synthesizes his own nicotinic acid, as evidenced by the work discussed above (34, 35, 38), under certain conditions it may have an important role in carbohydrate metabolism in this animal, for example Burke and McIntyre (40) showed that nicotinic acid markedly decreases insulin hypoglycemia in rats. Moreover, Ball (41) pointed out that at least three vitamins of the B complex, thiamin, riboflavin, and nicotinic acid, are collectively concerned in biological oxidations, and any investigation of the role of one of these demands a consideration of the other two.

No work has been published in which pure nicotinic acid or nicotinamide have been administered to adrenalectomized animals, and although it is indicated that no effects would be expected on its phosphorylation, the work discussed above (37, 40) would indicate an effect of nicotinic acid in adrenalectomy if the latter is, according to Laszt and Verzár, a secondary avitaminosis. Because of these possibilities, it was deemed of interest to include nicotinic acid in the present study.

*Experiment 2. Parenteral sodium nicotinate* (10/8/40). In a preliminary experiment involving 14 adrenalectomized rats of mixed sexes and 30 days of age, it was observed that subcutaneously administered sodium nicotinate was slightly toxic at 20

to 80 mg. daily doses. According to the paper of Unna (42), 315 mg. subcutaneously administered sodium nicotinate would be fatal to normal rats of the size used in this preliminary experiment. Adrenalectomy would be expected to increase its toxicity, as was actually indicated by the preliminary experiment, hence even lower doses than 20 mg. daily were used in the second experiment.

Sprague-Dawley female rats, 9 to 13 weeks old, were divided into 2 groups of 10 each, average body weight 133 (123–144) gm. Each group was caged together and fed a synthetic ration which contained: dextrinized starch 50, casein 20, yeast 10, salt mixture (28) 2, corn oil 9, and lard 7. This ration contained ca. 5 γ riboflavin and 20 γ thiamin per gm., and analyzed Na, 0.1%; K, 0.3%. Salt drinking fluid was offered for 24 hours after the operation. *Group 1* received 0.5 cc. saline subcutaneously from the operation until death, and group 2 received 10 mg. sodium nicotinate in 0.5 cc. $H_2O$ in the same way. *Group 1* survived 7.5 (4–11) days, and group 2, 6.1 (3–10) days. The average daily weight loss curves and food consumptions were similar in every respect.

It is concluded that large doses of nicotinic acid have no effect on weight maintenance, survival, or appetite of adrenalectomized rats on an adequate ration.

### 4. *Pyridoxine (Vitamin $B_6$) Therapy in Adrenalectomized Rats*

That carbohydrate and fat metabolism and transport are disturbed in adrenalectomy is well known. Fat metabolism, is also disturbed in pyridoxine deficiency.

Laszt and Verzár (43) stated that pyridoxine administered as a Fuller's earth adsorbate of yeast concentrate which had been acidified and autoclaved, had no beneficial effects on adrenalectomized rats, while whole yeast did. No experiments have been published in which pure pyridoxine in excess of dietary requirements, has been administered to adrenalectomized rats.

*Experiment 2. Parenteral pyridoxine* (9/9/40). Rats of the La Jolla strain, of mixed sexes, and 28 days old, were divided into 2 groups of 7 each, average body weight 50 (42–55) gm. Each animal was separately caged and fed the synthetic ration (see above). Daily food consumptions were recorded. Salt drinking fluid was offered for 24 hours following the operation. *Group 1* received 0.1 cc. saline subcutaneously from the day of the operation until death; while *group 2* received 10 mg. pyridoxine hydrochloride subcutaneously in 0.1 cc. saline daily until death. This dose is 18 times less than doses which are still tolerated by the rat (44). *Group 1* survived 3.8 (2–5) days, and *group 2* 4.2 (3–6) days. There was no difference in food consumption or average daily weight loss in the two groups.

A second experiment involving 14 rats, gave the same results when 5 mg. pyridoxine hydrochloride was administered daily by mouth.

It is clear that simple pyridoxine administration in excess of dietary requirement has no beneficial effect on adrenalectomized rats on an adequate diet.

### 5. *Filtrate Fraction (Pantothenic Acid, Factor W, and Vitamin Concentrate) Therapy in Adrenalectomized Rats*

Morgan and Simms (45, 46) noticed curative effects of adrenal cortical extracts in nutritional achromotrichia in rats, indicating a possible relationship between the adrenal cortex and this deficiency. Axelrod, Sober and Elvehjem (14, 15), in studying d-amino oxidase decrease in riboflavin deficiency in rats, found that with certain diets, there was an additive affect of Factor W or pyridoxine and riboflavin in restoring the liver oxidase to normal levels. They believe that it is possibly the protein component rather than the flavin component of the enzyme which is affected. Since it was indicated in the discussion on riboflavin, earlier in this paper, that the protein com-

ponent of the amino oxidase may also decrease in adrenalectomy, it was thought of interest to study possible effects of Factor W, pantothenic acid, and concentrates rich in filtrate fraction and the vitamin B complex in general, when administered to adrenalectomized rats fed an adequate diet containing 10% yeast, thus more than an adequate supply of the B complex.

*Experiment 1. Concentrate supplements. Grass concentrate* (7/15/40). Sprague-Dawley male rats, 33 days old, were divided into 2 groups of 5 each, average body weight 84 (75–91) gm., each group being caged together. Salt drinking fluid was offered for 24 hours after the operation. *Group 1* was fed the synthetic ration (see above) mixed with 'celluflour' in a 9:1 ratio. *Group 2* was fed the synthetic ration mixed with dehydrated, powdered cereal grass[8] in the same ratio. This product is canned under nitrogen and kept on ice. It is especially rich in vitamins, containing the following amounts of members of the B complex in mg. per gm.: riboflavin, 0.20–0.28; thiamin, 0.09–0.15; nicotinic acid, 0.75–1.50; $B_6$ complex in amounts such that 3–5% levels protect the rat from this deficiency; and the 'grass juice' factor and Factor W in "significant amounts" (47).

*Group 1*, the controls, survived 8.0 (4–13) days, and *group 2* survived 5.6 (5–6) days. The average daily food intake of *group 1* was slightly greater than that of *group 2*. Hence the grass concentrate slightly decreased survival and appetite rather than benefiting the rats. The potassium content of the control diet was 0.3%, while that of the diet with added grass concentrate was 0.8%, which probably explains the deleterious effect.

*Experiment 2. Concentrate supplements. Liver concentrate* (7/14/40). Sprague-Dawley male rats, 30 days old, were divided into 2 groups of 7 each, average body weight 72 (70–75) gm. Each rat was separately caged and fed. Salt drinking fluid was offered for 24 hours after the operation. *Group 1* received the synthetic ration, and *group 2* the synthetic ration, 95%, mixed with Wilson liver fraction #41725, 5%. This is an alcohol-soluble concentrate, kindly supplied by the Wilson Laboratories, Chicago, Illinois. It is substantially free of thiamin and pyridoxine, but containing per gram, 10–20 mg. nicotinic acid, 0.540 mg. riboflavin, 1.98 mg. pantothenic acid, and 159 units of 'filtrate factor' (assay furnished by Dr. David Klein of Wilson Laboratories). The Na and K contents were so low that at the 5% level used, no salt effects would be operative. *Group 1* survived 5.3 (5–6) days, and group 2, 3.8 (3–6) days. The average daily food consumptions and weight losses were the same in both groups.

*Experiment 3. Parenteral and oral pantothenic acid and pantothenic acid plus riboflavin* (6/25/40). Sprague-Dawley female rats, 30–40 days old, were divided into 3 groups of 6 each, average body weight 95 (90–104) gm. Each group was caged together and fed Rockland diet. Salt drinking fluid was offered for 48 hours before and after the operation. *Group 1*, the controls, received saline subcutaneously in the same amounts that were given to the other 2 groups. *Group 2* received 5 mg. calcium pantothenate in 0.5 cc. saline subcutaneously daily on the 2nd day after the operation, and thereafter for 3 days. After this they received pantothenate mixed in the food at a level of 300 $\gamma$ per gram for one day, thereafter the pantothenate therapy was discontinued. *Group 3* received 1.7 mg. riboflavin in addition to the 5 mg. pantothenate in 0.5 cc. saline on the 2nd day after the operation, and thereafter for 3 days; then 300 $\gamma$ panthothenate and 10 mg. riboflavin per gram of food for one day, thereafter the vitamin therapy was discontinued. *Group 1* survived 11.4 (8–17) days; *group 2*, 10.7 (6-13) days; and *group 3*, 6.7 (6–8) days. There were no differences in average daily food intake or weight loss.

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[8] Supplied by the courtesy of Cerophyll Laboratories, Kansas City, Mo.

*Experiment 4. Parenteral pantothenic acid and Factor W* (8/3/40). Sprague-Dawley male rats, 32 days old, were divided into 3 groups of 7 each, average body weight 72 (65–78) gm. Each rat was separately caged and fed the synthetic ration. Salt drinking fluid was offered for 24 hours after the operation. *Group 1*, the controls, received 0.5 cc. $H_2O$ subcutaneously daily. *Group 2* received 10 mg. pantothenate in 0.5 cc. $H_2O$ subcutaneously daily for 7 days after the operation; and *group 3* received a Factor W concentrate, 10 mg. of solid material in 0.25 cc. $H_2O$ daily for 7 days in the same way. This concentrate was supplied through the courtesy of A. E. Axelrod, of the University of Wisconsin, and was prepared from Wilson's liver extract (see *experiment 2*) by autoclaving with 1N $H_2SO_4$, neutralizing, and taken to dryness in vacuo. The neutral solution is low in both pyridoxine and pantothenic acid, but high in Factor W, 1 cc. per day being adequate for the requirements of a rat. Hence 10 mg. of the solid material represented a relatively massive supply of this factor.

*Group 1* survived 6.2 (4–7) days; *group 2*, 6.5 (5–9) days; and *group 3*, 6.4 (5–8) days. There were no differences in food intake or weight loss.

These experiments permit the following conclusions: If adrenalectomized rats are suffering a 'secondary avitaminosis' with respect to the several members of the B complex present in the substances used in the above 4 experiments, they are not benefited by massive administration of these substances. If members of the B complex such as Factor W and pantothenic acid, with or without riboflavin in relatively massive doses, have any effect in increasing the postulated decreased protein component of the amino oxidases of adrenalectomized rats' tissues, this effect did not benefit adrenalectomized rats with respect to survival, appetite, or weight maintenance when these rats are fed a diet which is adequate in all respects for a normal animal.

## SUMMARY

Relatively massive but non-toxic parenterally and orally administered thiamin hydrochloride and its pyrophosphate, cocarboxylase, exerted no beneficial effects on appetite, average daily weight loss, or survival of adrenalectomized rats maintained on a diet adequate for normal rats; nor was there a difference in action of thiamin and cocarboxylase.

The same conclusion was reached in the case of orally and parenterally administered riboflavin, and parenterally administered sodium phosphate ester of riboflavin in adrenalectomized rats and guinea pigs.

The same conclusion was made for parenterally administered sodium nicotinate, and parenterally and orally administered pyridoxine; for dietary supplements of a grass concentrate high in thiamin, riboflavin, nicotinic acid, Factor W, and the $B_6$ complex; and of a liver concentrate high in nicotinic acid, riboflavin, pantothenic acid, and filtrate fraction. Parenterally and orally administered pantothenic acid with and without massive doses of riboflavin, and parenterally administered Factor W concentrate likewise exerted no effects.

These results warrant the following conclusions. (*a*) If there is a relation between the adrenal cortex and thiamin or riboflavin, it is not because these substances are phosphorylated and subsequently effective only if the adrenal cortex is present, since the phosphorylated forms have no effect after adrenalectomy. (*b*) Excessive but non-toxic administration of most, if not all, of the members of the vitamin B complex has no effect on appetite, weight maintenance, clinical appearance, or survival of adrenalectomized rats fed diets which are adequate for normal rats. (*c*) The Laszt and Verzár theory that adrenal insufficiency is a 'secondary avitaminosis' is untenable in the light of these and other results.

Prior to receipt of proof of the present paper, J. W. Ferrebee (*J. Biol. Chem.* 136: 719. 1940) added confirmatory evidence to the work of Ochoa and Rossiter (6, 16) in demonstrating a lack of difference of phosphorylated thiamin and riboflavin in adrenalectomized rats' tissues.

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# THE EFFECT OF DESOXYCORTICOSTERONE ACETATE ON EARLY PREGNANCY[1]

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THAT the adrenal cortex produces an androgenic substance has been emphasized from time to time by numerous investigators, but definite proof was lacking until Reichstein (1) isolated an androgenic hormone, adrenosterone, from the adrenal cortex, which had one-fifth the potency of the male hormone, androsterone, and; more recently, Hooker and Collins (2) reported comb growth in capons after the use of desoxycorticosterone acetate.

Since the androgen, testosterone propionate, interrupted early pregnancy by causing the regression and resorption of the corpora lutea of early pregnancy (3), this investigation was undertaken to determine whether desoxycorticosterone acetate were sufficiently androgenic to similarly affect early pregnancy in mice.

The following report shows that desoxycorticosterone acetate will cause regression of the corpora lutea when the animals are injected on the first day of mating. If, however, injections are delayed until approximately 40 hours after mating, both the corpora lutea and embryos are supported—but only so long as injections are continued.

## PROCEDURE

Throughout these experiments, mated female albino mice were injected subcutaneously with 2 mg. desoxycorticosterone acetate (cortate) in an oily solution. These daily injections were begun either on the day of finding the vaginal plug or delayed until approximately 35 hours a.v.p. (after vaginal plug). Injections and autopsies were arranged as follows: *a*), injections begun the day of mating; autopsies not delayed; *b*), injections begun the day of mating; autopsies delayed; *c*), injections begun the day following mating; autopsies not delayed; *d*), injections begun the day following mating; autopsies delayed. As will be seen later, both the time of injection and the day of autopsy definitely affected the results.

## RESULTS

*a*). *Effects of injections begun the day of mating; autopsies not delayed.* Injections of 2 mg. cortate did not interfere with the progress of ova through the oviducts, as early embryos in pre-implantation stages were flushed from the uteri even after 3 daily injections.

The corpora lutea were prominent and vascular, showing increased luteinization in *mouse 54*, killed 60 hours a.v.p., and in *mouse 51* at 74 hours a.v.p. On the other hand, the corpora lutea of *mouse 52*, at 74 hours, were white.

The uteri were long, thin, quiet and hypoemic. Although the autopsies were performed prior to the implantation period, we doubt whether these uteri could have

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supported embryos because they differed so radically in appearance from normal organs at this stage.

*b). Effects of injections begun the day of mating; autopsies delayed.* The animals in this series were injected 2 to 8 times, and the autopsies were delayed 5 to 11 days a.v.p. to determine whether or not implantation occurred after the injections were discontinued. In no animals were implants found, but the fact that irregular blastocysts had been preserved in the uteri as many as 11 days a.v.p. is of particular interest.

In only one animal, *mouse 36*, was there any question about implants. Externally,

TABLE 1. EFFECTS OF DAILY INJECTIONS OF 2 MG. OF DESOXYCORTICOSTERONE ACETATE

| Animal No. | Treatment | Days a.v.p. | Condition of corpora lutea (c.a.=corpora albicantia) | Condition of embryos |
|---|---|---|---|---|
| | | *(a) Injections begun day of plug; autopsies not delayed* | | |
| 54 | 2/2/2/k | 60 hr. | Full; ovary pink. | Morula in uterus. |
| 51 | 2/2/2/k | 74 hr. | Vascular, enlarged. | Normal blastocysts in uterus. |
| 52 | 2/2/2/k | 74 hr. | Medium and enlarged c.a. | Blastocysts with zonas in uterus. |
| | | *(b) Injections begun the day of the plug; autopsies delayed.* | | |
| 48 | 2/2/-/-/-/k | 5 days | Enlarged, white. | Blastocysts in uterus. |
| 53 | 2/2/2/-/-/k | 5 days | Reduced, white. | Empty zonas and irregular blastocysts in uterus. |
| 35 | 2/2/2/2/2/ 2/2/2/-/k | 9 days | White, irregular. | Zona in tube. Contracted blastocyst in uterus. |
| 36 | 2/2/2/2/2/ 2/2/2/-/k | 9 days | Advanced, pink and mushroomed. | No eggs or implants. |
| 22 | 2/2/2/2/2/ -/-/-/-/-/k | 10 days | Small, pale and c.a. | Degenerate blastocysts in uterus. |
| 20 | 2/2/2/2/2/-/ -/-/-/-/-/k | 11 days | Small corpora albicantia. | Irregular blastocysts in uterus. |
| 21 | 2/2/2/2/2/-/ -/-/-/-/-/k | 11 days | Small corpora albicantia. | Irregular blastocysts in uterus. |
| | | *(c) Delay of 1 day before first injection; autopsies not delayed* | | |
| 41 | -/2/2/2/k | 108 hrs. | Vascular, overdeveloped. Follicles prominent. | Uterus beaded. |
| 42 | -/2/2/2/k | 109 hrs. | Large, vascular. | Uterus beaded. |
| 49 | -/2/2/2/2/k | 5 days | Large; pale, pink. | Uterus beaded. |
| 45 | -/2/2/2/2/2/2/k | 7 days | Pink. Much luteinization. | Implants. |
| 46 | -/2/2/2/2/2/2/k | 7 days | Vascular. | Implants. |
| | | *(d) Delay of 1 day before first injection; autopsies delayed* | | |
| 50 | -/2/2/2/2/-/-/-/k | 8 days | Regressed. Ovulation cones present. | Brown, loose embryos. Eggs with granulosa cells in ampulla. |
| 43 | -/2/2/2/2/2/ 2/-/-/-/-/k | 11 days | Albicantia reduced. | No eggs or implants. |
| 44 | -/2/2/2/2/2/ 2/2/-/-/-/k | 11 days | Regressed c.a. Follicles show signs of recovery. | Brown, macerated, detached embryos. |

the uterus showed several beads, but internally no embryos were visible, only thickened endometrium with small hemorrhage areas.

The corpora lutea showed various degrees of regression: those of *mouse 48* (injected twice, and killed on the fifth day a.v.p.) were large but white, while those of animals 20, 21 and 22 (injected 5 days and autopsied 10 or 11 days a.v.p.) were small corpora albicantia undergoing resorption. Only the corpora of *mouse 36*, the one with beaded uteri, were large and pink. From the results with animals injected on the day of mating, it appears that the corpora lutea are stimulated in growth up to about 72 hours, after which there is evidence of luteal regression.

Uteri were generally long and quiet, and some were thread-like. The uterus of *mouse 35* measured 3.5 cm. in length when first removed from the coelom. In *mouse 22* (5 days of cortate, delay of 5 days before autopsy) the uterus was more normal in

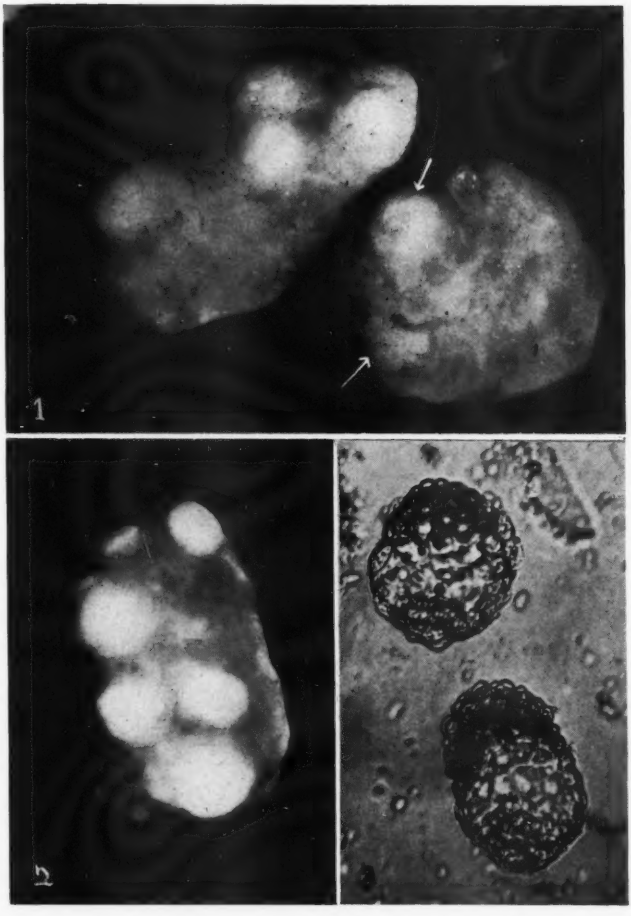

Fig. 1. OVARIES OF *mouse* 51, INJECTED 3 DAYS, STARTING THE DAY OF MATING, AND KILLED 74 HOURS a. v. p. The corpora lutea are large and still show signs of vascularity (dark stippling on the corpora lutea). ×17. Fig. 2. OVARY OF *mouse* 53, INJECTED 3 DAYS, STARTING THE DAY OF MATING, THEN KILLED AFTER A DELAY OF 2 MORE DAYS. The corpora lutea have definitely regressed and have become white corpora albicantia. ×17. Fig. 3. TWO OF 7 BLASTOCYSTS FLUSHED FROM THE UTERUS OF *mouse* 21, 11 DAYS AFTER MATING. This animal had been injected 5 times, beginning the day of mating, and autopsy was delayed until the eleventh day. Implantation usually takes place on the fourth or fifth day. The viability of such blastocysts has not been determined. ×225.

size and vascularity. Since the periovarial sacs were somewhat distended with fluid it is quite possible that this animal was returning to estrus.

*c). Effects of injections begun the day following mating; autopsies not delayed.* The significance of the time of injections was not appreciated until some of the preliminary data had been assembled, and even so, seemed to be without interpretation. However, as will be discussed later, the corpora lutea have undergone considerable development during the 35- to 40-hour delay.

In this series, all of the animals contained implants 4 to 7 days a.v.p.

The corpora lutea were, with but one exception, prominent vascular bodies, thus differing in appearance from those in series *b*.

The uteri were long and thin but more vascular than those of series *b*. Whether such uteri can continue to support embryos will have to be determined at another time.

d). *Effects of injections begun the day following mating; autopsies delayed.* Having established that both the corpora lutea and the embryos were preserved when injections were delayed 35 to 40 hours, it was questioned whether these would continue after injections were discontinued.

Accordingly, a few animals (50, 43, 44) were injected 4, 6 and 7 days and autopsied 8 and 11 days a.v.p. Seven dark, macerated embryos were flushed from the uteri of *mouse* 50 and no embryos were found in *mouse* 43, although the ovary contained regressed corpora lutea. Probably the embryos had been aborted. Again, in *mouse* 44 all the embryos were undergoing maceration and all but one were detached.

Not only were the embryos all dead in this series, but the corpora lutea had been reduced to corpora albicantia. It is not known which failed first, the corpora lutea or the uterus, but it would appear that desoxycorticosterone acetate had been directly or indirectly supporting the embryos. Another series of animals will have to be injected over a longer portion of the period of pregnancy to determine this point.

That the pituitary-ovarian relationship has not been seriously impaired after 4 days of injections is indicated by the fact that eggs with granulosa cells were discovered in the ampullae of *mouse* 50 8 days a.v.p., 4 days after the last injection.

*General effects.* Before closing these observations, it should be noted that continued injections of 2 mg. may have deleterious effects.

There was not only a reduced vascularity in the abdominal region of some animals, but the muscles and coelomic organs were abnormally brown in color.

## DISCUSSION

From these results, it is apparent that the effects of desoxycorticosterone acetate depend upon the time of the first injection. When this occurred on the day of the finding of the vaginal plug, the corpora lutea soon regressed, but when the first injection was delayed 35 hours a.v.p., there was increased luteinization and preservation of the corpora lutea. It will be recalled that mice usually mate at night; therefore ovulation had preceded, by several hours, the finding of the vaginal plug the following morning. If we may grant that ovulations probably occurred at least 5 hours before finding the plug, then there has elapsed not 35 but 40 hours between ovulation and the first delayed injection. This lapse of time is important in the development of the corpora lutea.

Since it is generally accepted that the amount of estrogen is low immediately following ovulation but greatly increases as the corpora lutea develop, it is probably the amount of estrogen present during this early critical period which controls the regression or maintenance of these bodies. While Allen and Bourne (4), Migliavacca (5), Winter, Reiss and Bálint (6) all report luteinization following the injection of cortical extracts, the experiments of Hohlweg (7) and Hoffman (8) best show the role of estrogen. Hohlweg reported that cortical substances caused luteinization in immature and castrated rats only after pre-treatment with estrogen. Hoffman found that luteinization followed the injection of a cortical extract only after the addition of some follicle stimulating hormone. According to our interpretation, Hoffman's results were made possible because of an increased production of estrogen by the activated ovaries.

If the estrogen does help preserve the corpora lutea in our delayed injection series, then it is clear why the corpora lutea soon fail in those animals injected on the

first day, when not only is there a reduced amount of estrogen then present, but its subsequent production is probably inhibited.

The quantity of estrogen present in the animal's body at the time of the first injection also probably controls the subsequent history of the embryo, as Evans (9) concludes from the work of Hisaw, Greep and Fevold (10) and Engle and Smith (11) that estrogen is very likely involved in implantation. Furthermore, Allen (12) clearly showed that estrogen must be present in the early days of both pseudopregnancy and pregnancy before a full proliferation response to injected progesterone can be obtained. In our experiments, implantation occurs with regularity only in the delayed injection series, after a sufficient time has elapsed for the uterus to be primed by estrogen from the developing corpora lutea. After this pre-treatment with estrogen, progestational effects are promoted either by the animal's own progesterone or by the progestational capacity of desoxycorticosterone acetate, or both. That this latter substance promotes progestational activity has been amply proven by numerous investigators (7, 13–18). However, in most of their experiments, progestational effects were best seen when the uterus had been pre-treated with an estrogen. Hohlweg (7), after using an estrogen, concluded that the activity of desoxycorticosterone acetate was one-tenth that of progesterone; Wells and Greene (15), having pretreated rabbits with estrone, obtained progestational effects in proportion to the amount of desoxycorticosterone acetate used; Heuverswyn, Collins, Williams and Gardner (16) found 6 to 10 mg. desoxycorticosterone acetate equal to 1 mg. progesterone in rabbits. When 1 mg. desoxycorticosterone acetate followed an estrone pre-treatment of 24 hours, 60% of their guinea pigs gave heat responses.

Although the role of estrogen seems established, our experiments, unfortunately, do not show the subsequent effects of cortate on the corpora lutea except in those series in which injections were not delayed. In the delayed injection series, where implants were noted, the corpora were prominent and red, even after 6 injections, but it is not known whether they were still functional, or whether they would regress if injections were continued. This raises the question whether desoxycorticosterone acetate preserves these corpora lutea, or whether they are kept from involution by products from the pregnant uterus or from the anterior pituitary.

One tentative answer is that desoxycorticosterone acetate has an additive progestational action, thus helping preserve the pregnant uterus, products from which, in turn, maintain the corpora lutea. When the injections of desoxycorticosterone acetate cease, the uterus can no longer support the embryos. This would indicate that either the corpora lutea are not functioning, or that the pregnancy is being carried along on amounts of progesterone greater than the corpora can produce alone. Although it now appears that repression of the corpora lutea in the delayed injection series is subsequent to the failure of the pregnant uterus, further investigations may prove the order of these events to be reversed.

On the other hand, there are other factors which may have a direct bearing on these results. Such would include changes in electrolyte balance, and atrophy of the adrenal cortex. Ingle and Kendall (19) and Ingle (20) report extensive atrophy of the cortex after large amounts of cortical extracts. Under these conditions, the adrenals could not function in the support of pregnancy after termination of the injections.

As was stated at the beginning of this report, the purpose of this investigation was to determine if there were certain androgenic properties in desoxycorticosterone acetate. We cannot answer this categorically. It is true that failure of the embryos to implant, and regression of the corpora lutea in those animals injected on the day of mating simulate the effects of testosterone propionate. Because of the absence of a delayed injection series in the androgen experiments, this part of the experiment

cannot be compared. However, we are inclined to believe that in these experiments desoxycorticosterone acetate was more andromimetic than androgenic in that, like testosterone propionate, it caused the regression of the corpora lutea of early pregnancy.

These results seem to indicate the importance of substances from the adrenal cortex in early pregnancy, and it may very well be that this gland is the source of the "extra-hypophyseal influence" maintaining the corpora lutea of early pregnancy for which Selye, Collip and Thomson (21) were searching.

## SUMMARY

The daily injection of 2 mg. of desoxycorticosterone acetate, begun the day of mating in albino mice, prevented embryo implantation. Blastocysts were flushed from long, thin, hypoemic uteri as late as 11 days after mating. Increased luteinization and vascularity were apparent up to approximately 72 hours after finding the vaginal plug, but later than this, regression of the corpora lutea was progressive. While these results duplicate those observed after injections of testosterone propionate, it is thought that desoxycorticosterone acetate, in these experiments, is andromimetic rather than androgenic.

On the other hand, if the first injection was delayed until the day following mating (approximately 40 hours after ovulation), embryos were implanted, and increased luteinization and vascularity were in evidence throughout the period of observation, 7 days. The difference in results between this and the first series is attributed to the production of sufficient estrogen in the delayed injection series to pre-treat the uterus before progestational conditioning.

However, the continued growth of embryos seemed dependent upon the progestational activity of desoxycorticosterone acetate. This is supported by the fact that withdrawal of this substance after implantation results in death of the embryos and regression of the corpora lutea.

The results are of further interest because they support the belief of numerous investigators that the adrenal cortex is an important factor in the performance of the ovary.

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# THE ABOLITION OF MATING BEHAVIOR BY HYPOTHALAMIC LESIONS IN GUINEA PIGS[1]

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DISTURBANCES of mating behavior and reproductive functions have been observed in 50 female guinea pigs with hypothalamic lesions (1). Twenty-three out of the 50 animals ran normal cycles so far as the changes in the ovaries, uteri and external genitalia were concerned (2) but they did not come into heat in the sense in which that word will be used in this paper, i.e., they did not show proestrual or estrual behavior and would not accept the male. Animals of this type will be referred to as Group I. In 12 others the genitalia were enlarged, the vaginae remained always open and the ovaries were highly follicular but without coporra lutea (Group II). In 7 the genitalia, including the ovaries, were atrophic and the vaginae remained always closed (Group III). Eight others ran very irregular cycles and showed changes resembling one or the other of the two groups last mentioned.

It is difficult to see how the failure to show estrual behavior could have been due to lack of ovarian hormones in the 23 animals which had normal ovaries. Furthermore, spayed guinea pigs with similar lesions failed to respond to injections of ovarian hormones in amounts greatly in excess of those sufficient to induce estrual behavior in the same spayed animals before the hypothalamic lesions had been placed (3). But before adopting the alternative hypothesis that the lesions in the brain were in themselves responsible for the behavioral abnormalities, it seemed desirable to make further attempts to overcome this abnormality by the injection of hormones. For this purpose a new series of animals was prepared.

## METHODS

The animals used in these experiments were young adult female guinea pigs weighing between 400 and 600 gm. Electrolytic lesions were placed in the brain with the aid of the Horsley-Clarke instrument by passing a direct current of 3 ma. for 30 seconds through a unipolar electrode. The coordinates were the same as those used in the earlier experiments already mentioned, the bare points of the otherwise insulated electrodes being placed as nearly as possible 1 mm. above the ventral surface of the brain at the level of the posterior border of the optic chiasma. Three lesions were made in each animal, one in the midline and one a millimeter on either side of the midline. In some animals control lesions were made in the same frontal plane and in the same relation to the midline, but at a level 6 mm. above the ventral surface of the brain and therefore above the level of the hypothalamus.

Some of the animals which received the lesions had been previously spayed, the others were normal. The latter were examined daily as to the condition of the external genitalia and vaginal membrane, and they were separated into 3 groups on the same

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basis as the original 50. The spayed animals had been brought into heat at least once before the placing of the lesions by properly timed injections of estrogen and progesterone (4). The estrogen[2] was injected in 4 doses of 25 I.U. on hours 0, 24, 48, and 60. The progesterone[3] (0.2 I.U.) was injected on hour 72; heat usually developed in from 4 to 6 hours and lasted 3 to 9 hours. The hormones, dissolved in oil, were injected subcutaneously.

For the determination of heat we have relied upon the methods described (5) modified to suit our particular purposes. For the elicitation of the estrual reflex we have utilized manual stimulation and the stimulation supplied by normal mature males. When attempts were being made to induce heat in the spayed females, the animals were stimulated at hourly intervals for a period of at least 12 hours following the injection of the progesterone. When attempts were being made to bring sterile unspayed animals into heat through the injection of ovarian or pituitary hormones the animals were kept under continuous observation from the time of opening of the vaginal membrane until at least 48 hours after the opening. The pituitary hormones used were follicle stimulating and luteinizing fractions prepared according to the method of Fevold (6) and supplied to us through the courtesy of Dr. W. H. Windle. A centrifuged suspension of fresh ground sheep anterior pituitary was also used in some of the experiments (7). The glands[4] were frozen at the time of removal and kept in a frozen condition until immediately before use. This saline extract was tested on immature female white rats. Four daily injections of 100 mg. eq. of the fresh glands caused canalization of the vaginae and 178 to 229% increase in the weight of the ovaries over the weight of the control ovaries.

## RESULTS

None of the animals with hypothalamic lesions came into heat. Some of the unspayed animals displayed normal cyclic changes in the genitalia (Group I); in others the genitalia hypertrophied and the vaginae remained continuously open (Group II); in still others the genitalia underwent atrophy and the vaginae remained continuously closed (Group III).

*Ovarian hormones.* These have been used on representatives of each of the 3 types. Five animals of Group I which ran regular cycles so far as the physical changes were concerned, were given subcutaneous injections from 12.5 to 25 I.U. of estrogen daily, beginning 4 days prior to the day upon which the vaginal membrane was expected to rupture. On the first morning after the membrane opened, progesterone (0.2 I.U.) was injected and the animals were tested each hour for at least 48 hours thereafter. None of these animals showed either proestrual or estrual behavior. Since the ovaries in animals of this type have normally developed follicles and corpora lutea (2) it seems probable that they secrete ovarian hormones and that the injected hormones would cause a high concentration of these substances in the blood. The normal ovarian function also speaks in favor of a fairly normal pituitary. It would seem probable therefore, that, following the injections, these animals had in their blood an excess of ovarian hormones and a fairly normal concentration of pituitary hormones. In spite of this they failed to come into heat.

Two animals of Group III whose vaginal membranes had been closed for over a month, and whose external genitalia showed signs of atrophy, were injected with 4 doses totaling 100 I.U. of estrogen followed on the fourth day by 0.2 I.U. of progesterone. The vaginal membranes opened for 2 and 3 days, respectively, but neither of the animals showed proestrual or estrual behavior.

[2] Theelin furnished through the courtesy of Dr. Oliver Kamm, Parke, Davis & Co.
[3] Prolution furnished through the courtesy of Dr. Erwin Schwenk, Schering Corporation.
[4] Kindly supplied by Dr. Edwin Pike, Armour & Co.

Eight animals of Group II with hypertrophied external genitalia and continuously open vaginae were given injections of progesterone, and in 4 the treatment was repeated a second time. In each case the vaginal orifices closed or became reduced to small slits within 4 days, then reopened after 3 to 8 days, but the animals showed neither proestrual nor estrual behavior. Three animals were killed 2, 3 and 4 days respectively after the vaginae closed following the second injection of progesterone. Luteinization had not occurred in the ovaries of any of these animals. These ovaries were not different from those previously described for this type of animal (2).

*Pituitary hormones.* It might be assumed that the combined action of some pituitary hormone and the ovarian hormones was a necessary condition for the mating reactions and that by reason of the proximity of the lesion to the hypophysis this

TABLE 1

| No. | Operation to progest. | Operation to estrus | Interval | Second trial | |
|---|---|---|---|---|---|
| | | | | Latent | Duration |
| | *hr.* | *hr.* | *days* | *hr.* | *hr.* |
| | | *High Lesions* | | | |
| 1 | 9 | 14.0 | | | |
| 2 | 12 | * | 10 | 3.0 | 6.0 |
| 3 | 12 | 16.0 | 10 | 4.0 | 6.0 |
| 4 | 12 | 17.0 | | | |
| 5 | 12 | * | 8 | 5.0 | 3.5 |
| 6 | 12 | * | 8 | 5.0 | 5.0 |
| | | *Low Lesions* | | | |
| 7 | 12 | * | 10 | * | 0 |
| 8 | 12 | * | 9 | * | 0 |
| 9 | 12 | * | 10 | * | 0 |
| 10 | 12 | * | | | |
| 11 | 12 | * | 8 | * | 0 |
| 12 | 12 | * | 8 | * | 0 |

* Failed to come into heat.

pituitary hormone was deficient in amount even in those animals with hypothalamic lesions which showed regular cyclic changes in the genitalia. If the lesions were placed shortly before the animal was due to come into heat the chance for the disappearance of this hypothetical hormone from the blood stream would be decreased. This line of reasoning was tested on a series of 12 spayed animals. Each animal was prepared for the elicitation of the estrual reflexes by the proper injection of estrogen. The animals were then lightly anesthetized with nembutal. In 6, lesions were placed in the usual position 1 mm. dorsal to the ventral surface of the brain, and in 6 others, 6 mm. dorsal to the ventral surface of the brain. The 12 animals were then injected with progesterone, at from 9 to 12 hours after the placing of the brain lesions. As far as could be determined, the general depression resulting from the high lesions was similar in duration to that resulting from the low lesions. The results of these experiments are shown in table 1. None of the animals with the low lesions, i.e., lesions in the hypothalamus 1 mm. above the ventral surface of the brain, came into heat during the period immediately following the operation nor after a second trial with the hormones several days later. On the other hand, 3 of the 6 animals with the high lesions 6 mm. above the ventral surface of the brain and above the level of the hypothalamus showed estrual behavior 14, 16 and 17 hours respectively after the placing of the lesions. The 3 refractory animals were tested again with the hormones some days later, and showed heat of normal duration after the usual latent period.

In so far as one may assume that any hypothetical hypophysial hormone would not have been likely to have disappeared from the blood in 16 or 17 hours following a hypothalamic lesion, the results of this experiment seem to indicate that the failure to show heat is not due to a deficiency of such a hormone. The experiments also show that the hypothalamic lesions have a specific effect not produced by similar lesions 5 mm. higher in the brain.

Luteinizing hormone was given to 5 of the animals of Group II with continuously open vaginae. Each received a single dose of 50 to 100 mg. eq. intraperitoneally. Following the injection the animals were observed every hour until the time of the closure of the vaginal membrane, and no proestrual or estrual behavior was observed at any time. The vaginal membranes closed within 2 days after injection and remained closed for the duration of a normal cycle. Two of these animals were killed for a histological study of their ovaries. It was found that the hormone induced luteinization in the ovaries, although the remnants of the ova and zona pellucida are discernible in some of the luteinized follicles. Two other animals subsequently received injections of chorionic gonadotropic hormone. Although the vaginal membranes closed for the duration of a normal cycle, the animals showed no signs of heat.

*Combinations of pituitary and ovarian hormones.* Luteinizing hormone, estrogen and progesterone were given to 6 animals, 2 of which belonged to Group II with continuously open vaginae. Three of the 6 were spayed animals with hypothalamic lesions; and one, a spayed animal without a brain lesion, served as a control. They were each given the customary treatment with estrogen. At the time of the last injection of estrogen, at the time progesterone was injected, and again at the end of the normal 4 to 5-hour latent period following the progesterone, luteinizing hormone (0.1 cc. or 82.3 mg. eq.) was supplied by intraperitoneal injection. Hourly observations were made for the following 36 hours, but no indications of estrual or proestrual activity were seen in any of the 5 animals with hypothalamic lesions. The control animal came into heat 5.5 hours after the injection of progesterone, showed estrous reflexes and copulated with a normal male.

Follicle stimulating and luteinizing hormones were given along with estrogen and progesterone to 6 animals, 3 of which belonged to Group I with regular cycles. One belonged to Group II with continuously open vagina, one was a spayed animal with hypothalamic lesions, and another a spayed animal with no brain lesion. Each animal was given a subcutaneous injection of 0.5 gm. eq. of FSH every other day for 6 days. Four subcutaneous injections of estrogen were made at the usual intervals. The pituitary and ovarian hormone injections were so timed in the animals of Group I that the vaginal membranes ruptured after the usual interval following their last openings. At the time of the last injection of estrogen luteinizing hormone was supplied by intraperitoneal injection and another dose was given along with the progesterone. None of the animals with the hypothalamic lesions showed either proestrual or estrual behavior; but the control animal showed estrual reflexes beginning 7.0 hours after the injection of progesterone and lasting for 3.5 hours.

Unfractionated pituitary extract, estrogen, and progesterone were administered to 5 spayed animals, 4 of which had lesions in the hypothalamus and one of which served as a control. Each received intraperitoneally 0.5 gm. eq. of a centrifuged saline extract of fresh anterior pituitary daily for 12 days. During the last 4 days, subcutaneous injections of estrogen and progesterone were injected in the usual manner. None of the experimental animals so treated showed either proestrual or estrual behavior. The control animal came into heat 6.5 hours following the injection of progesterone, and the heat lasted for 3 hours.

## DISCUSSION

There is strong evidence that the site of the erogenous action of the gonadal hormones is in the central nervous system, and not in the somatic and visceral structures which are also affected by these substances (8–12). Bard (13) states that it appears reasonable to assume "that an important central mechanism for estrual behavior one which is specifically activated by estrin must be situated somewhere in the territory comprising the preoptic and septal areas, a part of the thalamus, the subthalamus, the hypothalamus and the upper portions of the midbrain." We have shown that properly placed lesions in the hypothalamus abolish estrual behavior in the guinea pig. Because of the close topographic relation of the hypothalamus with the hypophysis and the dependence of the ovaries on the hypophysis it seemed possible that the behavioral abnormality might be dependent on a deficiency of some hypophysial or ovarian hormone. But this idea has received no support from the experiments reported in this paper. Guinea pigs with hypothalamic lesions which have received hypophysial and ovarian hormones in quantities sufficient to overcome any such deficit have not come into heat. It seems therefore highly probable that the lesions seriously damaged the central mechanism for estrual behavior which, according to Bard, is specifically activated by estrin.

Dempsey and Rioch (14) place the integrating mechanism for estrual behavior in the posterior part of the hypothalamus at the level of the mammillary bodies and Bard (13) presented evidence that in the cat it may be situated in the rostral part of the mesencephalon. The lesions in our guinea pigs have been in the anterior part of the hypothalamus. Further research will be required to determine the exact location and boundaries of this central mechanism.

It is important to emphasize, however, that our guinea pigs with hypothalamic lesions were in excellent health and could be kept under observation as long as desired. Some of the animals in the first group of 50 were observed for 6 or 7 months.

## CONCLUSIONS

Guinea pigs with properly placed lesions in the anterior part of the hypothalamus failed to show proestrual or estrual behavior and such behavior could not be induced in them by the injection of ovarian hormones alone or in combination with pituitary hormones. The failure of such hormones to bring these animals into heat increases the probability that the behavioral difficulty is due to impairment of a central mechanism for estrual behavior.

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# THE METABOLISM OF DOGS WITH PERMANENT DIABETES PRODUCED BY ANTERIOR PITUITARY EXTRACT

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IN A RECENT PUBLICATION (1) we recorded our observations upon the induction, course and pathology of the permanent diabetes that may follow a series of injections of anterior pituitary extract in dogs. In this paper we present the results of studies upon: (*a*) the relationship of the concentration of the blood sugar and serum fatty acids to the severity of the diabetes, insulin administration and variations in diet, (*b*) the urinary excretion of glucose, nitrogen and ketone bodies under the same circumstances and (*c*) the respiratory metabolism. An effort has been made to present data which supplement those already published.

Marks and Young (2) have recently summarized their observations upon various aspects of this type of experimental diabetes to which they have given the name pituitary-diabetes.

## METHODS

*General.* The data included in this paper were obtained from observations on male dogs throughout the course of the permanent diabetes that followed a period of intraperitoneal injections of crude saline anterior pituitary extract. Except where indicated the separate studies in this paper do not necessarily represent coincident observations on the same animal. Details concerning the induction of the diabetes, collection of urine, and feeding and food analyses have been published (1).

In order that observations upon the blood sugar, serum fatty acids and respiratory metabolism might be correlated with the increasing severity of the diabetes the entire course was divided into consecutive 3- to 5-day periods and the glucose excreted in the urine during each period was determined by daily analysis. The urinary glucose excretion has been expressed as a percentage of the available glucose of the (ingested) diet and this has been used as an approximate index of the severity of the diabetes. It has been assumed to represent the general degree of severity at the time of any observation made within that period. The calculation and the significance of the "per cent of the available glucose of the diet excreted" has already been considered (1). Apparently it is not affected by moderate variations in food intake, and it has a wider application than fasting glycosuria which only occurs in severely diabetic animals. In our dogs the intensity of untreated diabetes progressively increased until over 80% of the available glucose of the diet was excreted. In three dogs (P22, P26 and P36) in the absence of insulin therapy the fasting urinary excretion of glucose and nitrogen was of the same order as that of totally depancreatized dogs under similar conditions (1). Diabetes of this severity has been called total urinary diabetes.

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*Chemical.* Blood samples were taken between 8:30 and 11:30 A.M., 16 to 24 hours after the last feeding, from the veins of the ear or neck. The urine glucose was determined by the Benedict method, urine and food nitrogen by the micro-Kjeldahl and urine ketones (expressed as acetone) by the Van Slyke method. A modification of the method of Stoddard and Drury was employed for the determination of the serum fatty acids. Blood glucose was determined by a modification of the Folin micro-method. With a few exceptions, the determinations of urinary nitrogen and ketones were made in duplicate.

*Respiratory metabolism* was determined in a closed circuit apparatus of the Benedict 'universal' type (3). The capacity of approximately 250 liters was, in later experiments, reduced to 152 liters by placing a varnished wooden block in the chamber, thus reducing the error in correcting the volume for barometric pressure change. A water jacket maintained the temperature of the animal chamber constant at about 25°C., care being taken to terminate an observation at the same temperature as that initially observed. The stream of air was not resaturated with water vapor before entering the chamber.

The residual gases in the apparatus were not routinely determined. The necessity for this was avoided by instituting a foreperiod of about an hour, at the end of which the concentration of gases in the chamber was shown to have assumed a value which remained constant throughout the experimental period immediately following. With an alcohol lamp burning in the chamber, samples of air were taken at the beginning and end of the second hour following the start of ventilation. Analysis in a Haldane-Henderson apparatus indicated a change in carbon dioxide concentration of 0.03% and in oxygen of 0.04%. Such changes are barely outside the limit of experimental error of the analysis. Inasmuch as the rate of oxygen utilization of the burning alcohol was considerably greater than that of a dog, the change in the residual gases would be even less in the latter case. Thus all the carbon dioxide absorbed and oxygen used were solely a result of the animal's metabolism and not of changing the gaseous composition of the residual air.

Thirteen alcohol checks were performed at various intervals during the course of our experiments. The average R.Q. of these was 0.666 with variations from 0.650 to 0.680. The alcohol burned was not measured so that total $CO_2$ production and oxygen consumption were not checked. The results are believed to be accurate on a comparative basis in spite of the omission of this control, since an identical routine was maintained for all the experiments.

In the majority of cases, basal observations were made 20 to 24 hours after the last food (and insulin, if administered), but in a few instances as early as 18 or as late as 28 hours. In the observations upon the effect of food the interval between the ingestion of glucose or meat and the start of the measurement of the respiratory exchange varied between 1 and 4½ hours. When glucose was given before the test the amount varied between 25 and 100 gm., and in the case of meat was equal in amount to that contained in the dog's daily diet as indicated in the legend of figure 5. Ten units of insulin were given with glucose in the tests so charted. In dog P23 when insulin was given before the basal test the dose varied from 4 to 12 U.

To avoid the danger of urinary tract infection, which may occur in male dogs, urine collections were not made by catheterization. Before and after the usual 2½-hour period during which the dog was in the metabolism chamber it was placed in a run-way where it usually voided and the urine was collected in a pan. The completeness of the voiding was determined by palpation of the bladder, and incomplete specimens were discarded. Although this method can give only a rough approximation of the nitrogen excretion it sufficed to indicate a definite relationship between

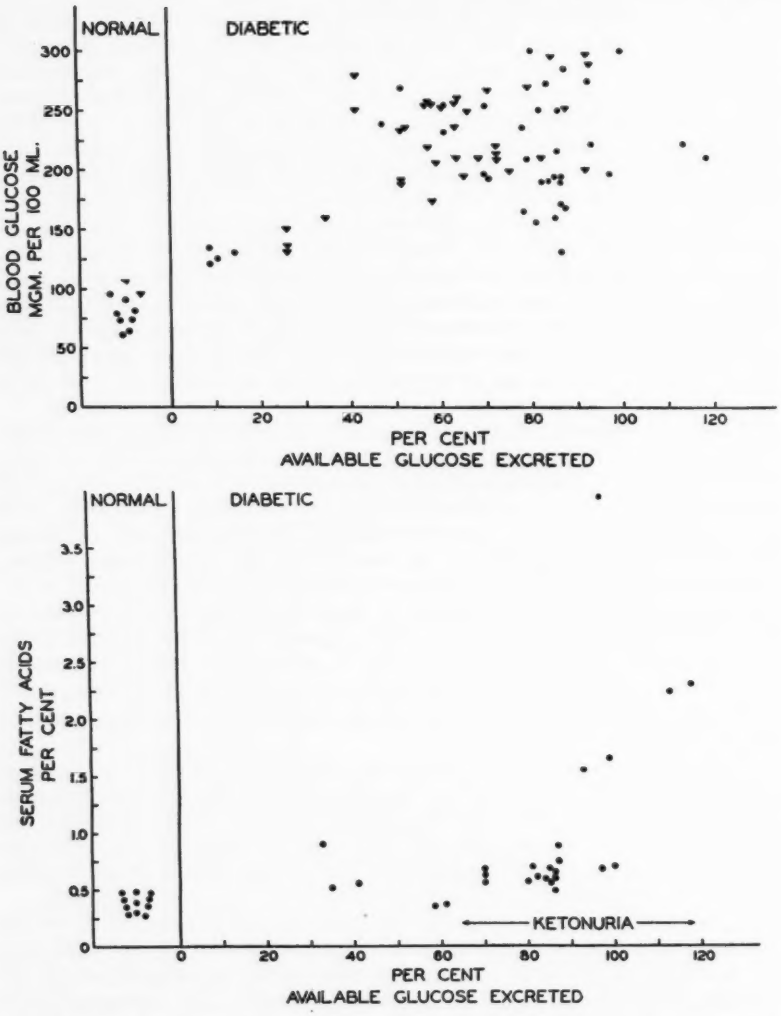


Fig. 1. The relation of the blood glucose concentration to the severity of the diabetes in dogs, not receiving insulin therapy. Observations were made 16-24 hours after the last feeding. The severity of the diabetes is indicated by the proportion of the available glucose of the diet excreted during the 3- to 5-day period in which the observation was made.

● Dogs receiving a meat diet (400 to 1000 gm. of meat, average available glucose = 91 gm. per day).
▼ Dogs receiving a meat and sucrose diet (average available glucose = 177 gm. per day).

Fig. 2. The relation of the concentration of serum fatty acids to the severity of the diabetes in dogs not receiving insulin therapy. Observations were made 16–24 hours after the last feeding. Data from all dogs eating a daily diet of 400 to 1000 gm. of beef, with or without added sucrose The severity of the diabetes is indicated by the portion of the available glucose of the diet excreted during the 3 to 5-day period in which the observation was made. The individual animals varied in the severity of diabetes (65–93%) exhibited at the time of the onset of persistent ketonuria.

urinary nitrogen and the oxygen consumption. However, individual observations were not accurate enough to allow calculation of the non-protein respiratory quotient.

## RESULTS

### *Blood Glucose and Serum Lipids*

*The relationship of the fasting blood glucose value to the severity of the diabetes* (fig. 1). It is evident that when the diabetes was mild the fasting blood glucose was only slightly elevated above normal values. However, if the excretion of available

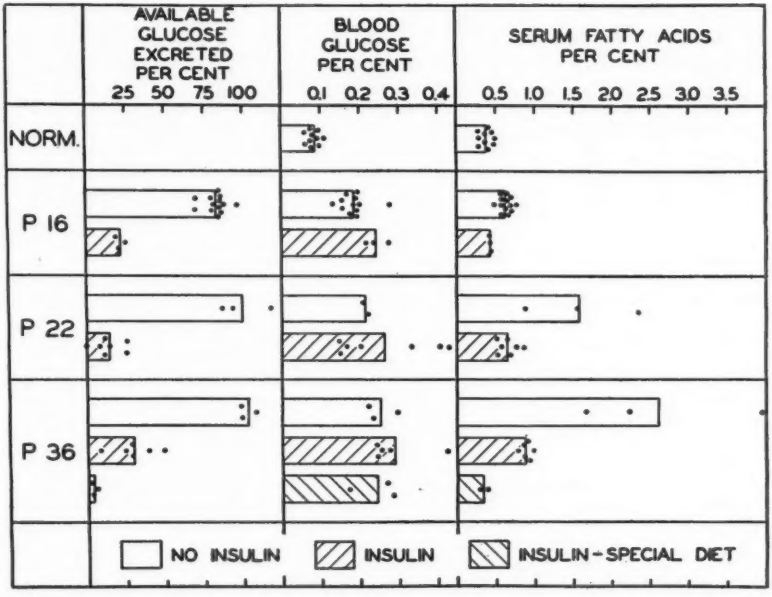


Fig. 3. Effect of insulin upon the blood glucose and serum fatty acids in severely diabetic dogs. The determinations were made 16–24 hours after the last food (and insulin) in dogs excreting approximately 80% or more of the available glucose of the diet without insulin. The first column indicates the proportion of available glucose excreted during the 3 to 5-day period in which the observation was made. The special diet consisted of 200 gm. of beef plus sucrose. The diet in all other instances contained 500 to 1000 gm. of beef with or without added sucrose. Three U. of protamine zinc insulin were given to dog P36 the afternoon before the determinations on the special diet and twice during periods of regular diet. *Dog P16* received 2 U. of protamine zinc insulin on one occasion, otherwise only regular insulin was given on the day preceding the observation. When insulin was administered it was given for 3 or more days before a sample was obtained.

glucose of the diet exceeded about 40% there was in general a higher concentration of blood glucose, associated with such a wide variation that there was a poor correlation with the severity of the diabetes as determined by this approximate index.

The high blood glucose values 16 to 24 hours after the last food and insulin during periods of insulin treatment as shown in figure 3 is further evidence of the poor correlation of the fasting blood glucose with the percentage of available glucose of the diet excreted. There was also no correlation between the concentration of the serum fatty acids and the blood sugar determined on the same samples.

*The relationship of the fasting serum fatty acids to the severity of the diabetes* (fig. 2).

Four of the five values obtained during periods in which less than 65% of the available glucose of the diet was excreted were within the normal range or nearly so. The one high value during this stage was found in *dog P23* which at autopsy had interstitial nephritis. As the diabetes became more severe there was an increase in the level of the fatty acids so that when more than 90% of the available glucose was excreted the serum fatty acids were over three times the maximal normal value in 5 of the 7 instances. Persistent ketonuria did not develop until the excretion of the available glucose of the diet was 65% or more and it was not until this stage that a persistent increase in the concentration of the serum fatty acids was observed. Despite this there was no correlation between the amount of ketone bodies excreted and the concentration of the serum fatty acids in individual instances (see below).

TABLE 1. THE SERUM FATTY ACIDS AND KETONURIA OF PITUITARY-DIABETIC DOGS DURING PERIODS OF FASTING AND FAT OR MEAT FEEDING

| Dog No. | Time from last ant. pit. inj. to period | Duration of period | Serum fatty acids | | | Urine ketones during period |
|---|---|---|---|---|---|---|
| | | | 1[1] | 2[1] | 3[1] | |
| *Dogs with total urinary diabetes*[2] | | | | | | |
| *Fasting* | | | | | | |
| | days | days | % | % | % | gm./day |
| P22 | 78 | 3 | 0.50 | 0.64 | — | 0.01 |
| | 124 | 5 | — | 0.72 | 0.90 | 1.01 |
| | 180 | 5 | 0.57 | 0.69 | 0.76 | 0.08 |
| | 200 | 5 | 0.65 | 1.15 | 1.11 | 0.12 |
| | 385 | 5 | 0.86 | — | 0.42 | 0.18 |
| P36 | 96 | 5 | 0.78 | — | 0.44 | 0.28 |
| | 201 | 5 | 0.99 | 0.77 | 0.89 | 2.58 |
| *Fat Diet* | | | | | | |
| P22 | 90 | 5 | — | — | 2.22 | 0.56 |
| | 145 | 5 | 0.68 | 4.30 | 9.28 | 0.30 |
| P36 | 149 | 5 | 0.91 | 4.75 | 3.68 | 1.94 |
| *Meat Diet* | | | | | | |
| P22 | 101 | 3 | 0.50 | 2.30 | — | 3.19 |
| P36 | 242 | 8 | 0.90 | 2.23 | 1.66 | 5.76 |
| *Dog Excreting About 80% of Available Glucose of Diet*[2] | | | | | | |
| *Fasting* | | | | | | |
| P16 | 157 | 7 | 0.68 | — | 0.42 | 0.00 |
| | 303 | 7 | 0.62 | 0.48 | 0.53 | 0.03 |
| *Fat Diet* | | | | | | |
| P16 | 571 | 16 | 0.56 | — | 0.52 | 0.12 |
| | 689 | 5 | 0.59 | 0.52 | 0.49 | 0.11 |

[1] Blood samples were obtained on (1) the 1st day of the period and (3) at the end of the last day. The sample (2) was taken at the end of the third day in 5 day periods and at the end of the fourth day in the 7 and 8-day periods. The last sample of the 3 day period is placed in column number 2.

[2] In dogs with total urinary diabetes the last dose of insulin was given the evening before the period began. In *dog P16* no insulin was given before or during the period.

The normal values for serum fatty acids varied from 0.29 to 0.50%.

It has been previously observed (4) that during the period of injection of anterior pituitary extract the serum fatty acids are increased. In our dogs the average of 11

observations (not charted) during the period of injection was 72% greater than the average value obtained in 11 specimens from non-diabetic animals, and the individual observations exceeded the maximum normal values in all but one instance.

*The effect of insulin treatment upon the serum fatty acids and blood sugar* (fig. 3). The serum fatty acids were reduced in all 3 dogs during periods of insulin therapy. During insulin treatment in the case of *dog P16*, the serum fatty acid values were within normal limits as were those obtained in *dog P36* upon the diet of 200 gm. of meat plus sugar. That this reduction of beef intake alone was not responsible for the

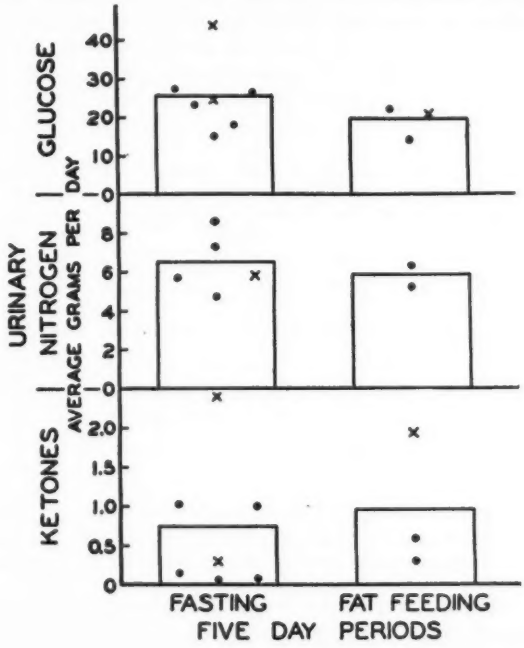


Fig. 4. THE AVERAGE DAILY URINARY EXCRETION OF GLUCOSE, NITROGEN AND KETONES (expressed as acetone) DURING 5-DAY PERIODS OF FASTING OR FAT FEEDING IN 2 DOGS WITH "total urinary diabetes." The last regular feeding and insulin was given about 17 hours before beginning urine collections. Nitrogen determinations were omitted in 3 of the periods.
● = *Dog P22*—received an average of 73 gm. of lard per day.
X = *Dog P36*—received an average of 115 gm. of lard per day.

decrease to normal levels was suggested by the effect of fasting (noted below) and by uncharted observations showing a lack of significant effect from a similar diet in the absence of insulin therapy in *dog P16*.

*The effect of fasting and fat or meat diets upon the serum fatty acids when no insulin was given* (table 1). It may be noted that the serum fatty acids of the two dogs with total urinary diabetes exhibited a variety of responses to fasting. There was no evident explanation for this variability. During the period of fat feeding both dogs exhibited a marked increase in the serum fatty acids and a less marked rise during the period of beef ingestion. A value of 24.7% was obtained in *dog P22* at the end of fasting day which followed the 5 days of fat feeding beginning on the 145th day.

In the dog excreting about 80% of the available glucose of its diet, fat feeding as

well as fasting was associated with a slight decrease in the serum fatty acids. In this as in the totally diabetic dogs an average of more than 70 gm. of fat a day was ingested. The average daily excretion of ketone bodies during these periods is recorded in the last column. It is clear that there is no correlation between the serum fatty acid values and the quantity of ketone bodies excreted.

*Miscellaneous observations.* The concentration of cholesterol in the serum was determined in 30 instances. The Liebermann-Burchard reaction was employed. In general the cholesterol concentration in the serum of diabetic animals was above that of normal dogs and the changes incident to the use of insulin and dietary variations were in the same direction as those exhibited by the serum fatty acids. However, the proportional changes were usually much less than those of the serum fatty acids. Similar results have been reported in cases of human diabetes mellitus (5).

The concentration of $CO_2$ in the serum was determined in a few instances. In the dogs that did not have total urinary diabetes the values were within normal

TABLE 2. THE AVERAGE DAILY URINARY EXCRETION OF GLUCOSE, NITROGEN AND ACETONE BODIES IN DOGS WITH TOTAL URINARY DIABETES (MEAT FED NO INSULIN)

| Dog No. | Period | Body weight | | Av. meat ingested | Glucose | | Nitrogen | | Ketones | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Initial | Av. loss | | In urine | In food[1] | In urine | In food[1] | In urine | In food[1] |
| | *days* | *kg.* | *kg./day* | *gm./day* | *gm./day* | *gm./day* | *gm./day* | *gm./day* | *gm./day* | *gm./day* |
| *P22* | 3 | 8.75 | 0.20 | 635 | 84.8 | 68.5 | 24.2 | 17.5 | 3.19 | 37.1 |
| *P36* | 4 | 12.00 | 0.34 | 525 | 64.3 | 56.7 | 19.7 | 14.5 | 4.03 | 30.9 |
| | 4[2] | 10.15 | 0.08 | 505 | 54.3 | 54.5 | 15.6 | 13.9 | 6.49 | 29.4 |

[1] See methods and results for technic of calculations.
[2] This period immediately followed the one above.

limits despite moderate ketonuria which in one case reached 1.7 gm. (as acetone) during the 24 hours preceding the observation. In a dog with total urinary diabetes a value of 21 vol. % was obtained after withholding insulin and food for 3 days.

### *Urinary Excretion of Glucose, Nitrogen and Ketones*

*A comparison of the effect of fasting and fat feeding in dogs with total urinary diabetes during periods without insulin therapy* (fig. 4). It will be noted that the average excretions of glucose, nitrogen and ketone bodies are of about the same magnitude under both circumstances. The initial body weight of *dog P22* varied from 8.75 to 10.35 kg. and that of *dog P36* from 11.35 kg. to 12.50 kg. During the periods of fasting the average daily weight loss was 0.25 kg. and 0.24 kg. and during fat feeding was 0.13 and 0.19 kg. for dogs *P22* and *P36* respectively.

*The effect of the ingestion of meat, in dogs with total urinary diabetes, during periods without insulin therapy* (table 2). In these animals, which had the fasting urine glucose and nitrogen excretion of totally depancreatized dogs (1), there was a negative nitrogen balance and a glucose excretion greater than that attributable to the meat ingested. The acetone body excretion was considerably greater than the largest amount (2.58 gm. per day) excreted during the periods of fasting or fat feeding, even though the calculated ketogenic material available for the production of acetone bodies from the fat and protein of the meat was considerably less than the 56 gm. available when the fat diet was ingested. In all instances the excretion of acetone bodies was far less than the calculated possible production if all ketogenic material had been converted. The calculations were made using the usual conversion factors except that it has

been assumed that 4 molecules of aceto-acetic acid may be formed from each molecule of fatty acid. That such an assumption is probably warranted has recently been emphasized by the work of Stadie, Zapp and Lukens (6). The weight loss during the first 3 days was similar to that found in these animals during fasting. This is in contrast to the somewhat smaller loss of weight during fat feeding.

*Studies in a pituitary-diabetic dog excreting about 80% of the available glucose of the diet* (dog P16) (table 3). Observations previously published (7) show that in the mild or moderately diabetic dog fasting and fat feeding were associated with little or no glycosuria and ketonuria and that there was little or no increase of nitrogen excretion above that found in normal dogs. Similar results were obtained in this more

TABLE 3. AVERAGE DAILY URINARY EXCRETION OF GLUCOSE, NITROGEN AND ACETONE BODIES OF *Dog P16*[1] DURING PERIODS WHEN INSULIN WAS NOT ADMINISTERED

| Diet | Duration of period | Weight | Urine | | |
|---|---|---|---|---|---|
| | | | Glucose | Nitrogen | Ketones |
| | | | Average and range | | |
| gm./day<br>Meat 1000 | days<br>* | kg.<br>12.6 | gm./day<br>90.4<br>83.6–93.4 | gm./day<br>28.4<br>26.7–31.0 | gm./day<br>0.34<br>0.25–0.53 |
| Meat 1000<br>Sucrose 100 | † | 13.6 | 177.0<br>173–180 | 26.8<br>26.8–26.8 | 0.31<br>0.23–0.40 |
| Sucrose 100 | 3 | 12.7 | 76.7 | 5.0 | 0 |
| Fat 75 | 5 | 12.5 | 1.3 | 4.2 | 0.12 |
| Fasting | 5 | 12.3 | 0.9 | 3.4 | 0.04 |

[1] Observations made between the 200th and 310th day after the last injection of anterior pituitary extract. Approximately 80% of the available glucose of the meat or meat and sucrose diet was excreted.
* Average of 11 periods of 3 to 5 days each.
† Average of 2 periods of 4 and 5 days.

severely diabetic dog. It may be calculated from table 3 that proportions of the available glucose of the diet excreted in the urine were about the same when ingesting diets composed of meat or sucrose only, or both together.

### *Respiratory Metabolism*

The results have been expressed in figure 5 as liters of oxygen (at N.T.P.) per square meter of surface area per hour. The Cowgill-Drabkin formula (8) for surface area has been employed. The oxygen consumption may be used as an approximate index of heat production but in considering the significance of differences one must allow for the increase of about 8% in the calorific value of oxygen as the respiratory quotient increases from 0.7 to 1.0. The results have been analyzed by the small sample method of Fisher. Those differences stated to be significant have a p value of less than 0.05.

*Basal oxygen consumption.* The data presented in figure 5 show that the average oxygen consumption of *dog P23* during a period of untreated diabetes was significantly increased (19%) above that observed before the animal was made diabetic. The diabetic dogs *P22* and *P16* had an average basal oxygen consumption 9.9 and 9.4% greater than the non-diabetic basal oxygen consumption of *dog P23*. The interpretation of this is uncertain, but it may be noted that the oxygen consumption of our non-

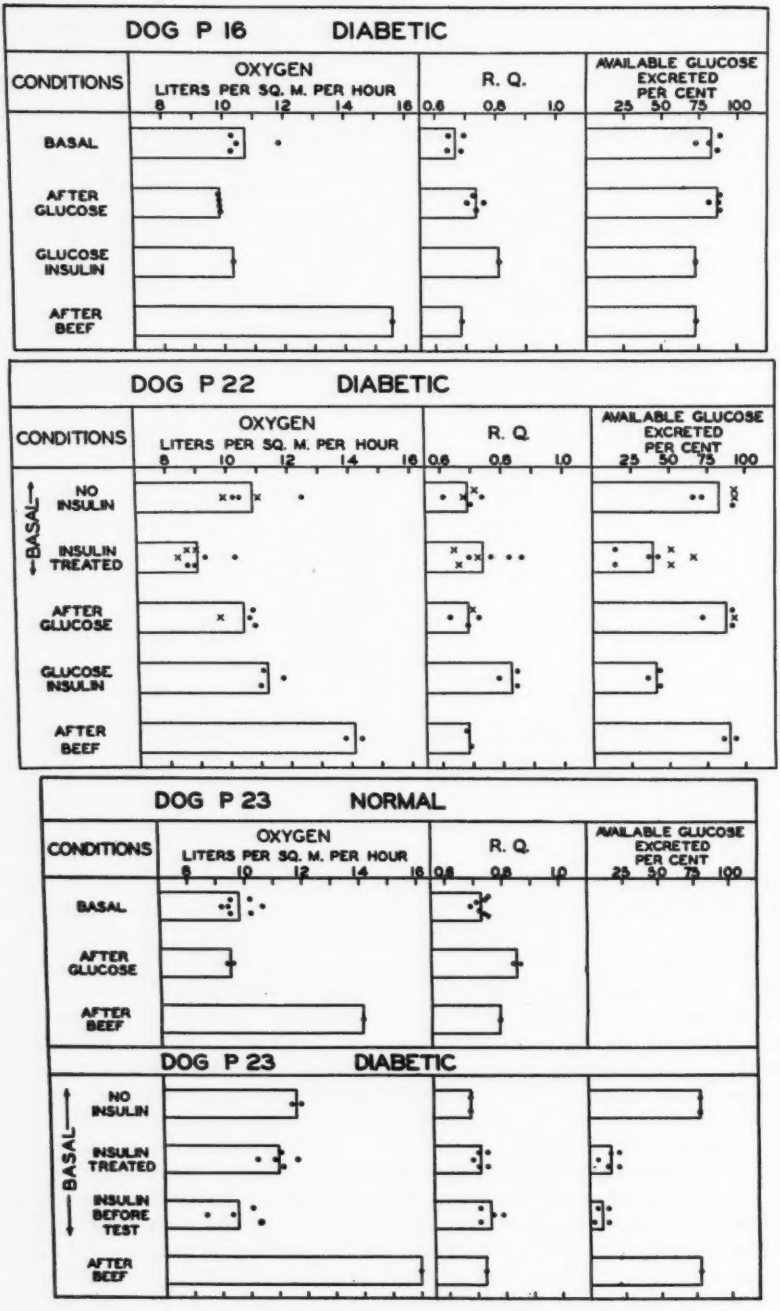


Fig. 5 (See opposite page for legend.)

diabetic dog is in the upper range of the normal figures given by De Beer and Hjort (9) whose results are also based upon the Cowgill-Drabkin surface area formula.

*Effect of insulin upon oxygen consumption.* The average basal oxygen consumption of *dog P22* when not treated with insulin was significantly increased by 21% above the average of the observations made during the period of insulin treatment. In *dog P23* the effect of insulin upon the oxygen consumption was more apparent when insulin was given a few hours before the tests (during the periods of insulin treatment). There was, under these circumstances, a significant reduction in oxygen consumption to within 2% of that observed before the dog was made diabetic.

The results following the injection of insulin during a fasting experiment of 113 hours are shown in table 4. At the 90th hour after the last food and insulin there was

TABLE 4. THE RESPIRATORY METABOLISM OF *Dog P22* DURING A FASTING PERIOD

| Interval since food | Interval since insulin | R. Q. | Oxygen consumption | | | Weight |
|---|---|---|---|---|---|---|
| *hr.* | *hr.* | | *l./hr.* | *l./m².hr.* | *l./kg./hr.* | *kg.* |
| 19 | 19 | 0.82 | 3.92 | 8.88 | 0.467 | 8.40 |
| 90 | 90 | 0.66 | 4.23 | 10.02 | 0.568 | 7.45 |
| 94 | 1[1] | 0.67 | 4.03 | 9.56 | 0.541 | 7.45 |
| 113 | 1[1] | 0.71 | 3.62 | 8.62 | 0.496 | 7.30 |

[1] Ten units of insulin were given 1 hour before the observation.

a slight increase in total oxygen consumption. One hour following insulin injections there appears to have been a significant decrease in the oxygen consumption at the 94th hour and again at the 113th hour.

*Effect of glucose upon the oxygen consumption.* This was not found under the conditions of our experiments. However, the administration of glucose and insulin to *dog P22* was associated with an increase in oxygen consumption of 23% above the average basal oxygen consumption during the period of insulin treatment.

*Ingestion of meat.* This was followed by a marked rise in oxygen consumption in all cases. Quantitative comparisons cannot be made because of the few observations and the variation in the amounts of meat ingested.

*Basal respiratory quotient.* The average R.Q. of *dog P23* while receiving a meat diet before being made diabetic was 0.73 and after the production of diabetes was 0.68 when not receiving insulin treatment. Under similar circumstances the average basal R.Q. of diabetic *dog P16* was 0.67 and that of diabetic *dog P22* was 0.69.

*Effect of insulin, glucose and meat upon the R.Q.* In both dogs treated with insulin the average R.Q. approximated that found in normal dogs receiving a similar diet.

Following the ingestion of glucose, *dog P16* exhibited a significant increase of the average R.Q. to 0.73, but in *dog P22* no change was observed. As expected, *dog P23* had a marked rise in respiratory quotient following glucose ingestion before the induction of diabetes. Following glucose and insulin both the tested diabetic dogs had approximately the same R.Q. as that found in the non-diabetic dog after glucose alone.

Fig. 5. THE OXYGEN CONSUMPTION AND RESPIRATORY QUOTIENT OF ONE NORMAL AND THREE PITUITARY-DIABETIC DOGS.

The proportion of the available glucose of the diet excreted in the urine, during the 3 to 5-day periods in which the observations were made, has been included to indicate the approximate severity or degree of control of the diabetes.

Diets: *P16*: 1000 gm. of beef.
*P22*: 750 gm. of beef = **X**, 750 gm. of beef and 75 gm. of sucrose = ●.
*P23*: 750 gm. of beef.

Diabetic dogs *P16* and *P22* showed no significant change in the R.Q. following the ingestion of beef. This is in contrast to the result of 0.79 obtained in dog *P23* before being made diabetic. In this dog the R.Q. was 0.73 following beef ingestion during a period of untreated diabetes.

*The relationship of urinary nitrogen excretion and oxygen consumption* (fig. 6). In those instances in which urine collections were made a definite relationship between the nitrogen excretion and oxygen consumption is evident in spite of the relatively large errors inherent in our method of urine collection. The observations which were

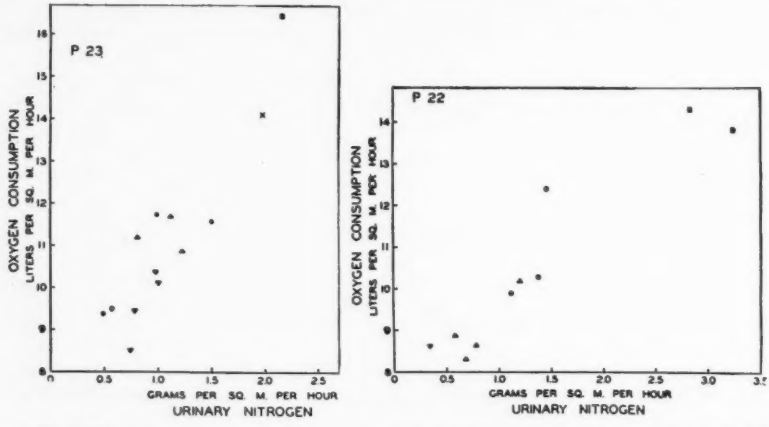


Fig 6. THE RELATIONSHIP OF URINARY NITROGEN EXCRETION AND OXYGEN CONSUMPTION. ● Basal—no insulin. ▲ Basal—insulin treated. ▼ Basal—insulin treated and insulin before test. ■ After beef. ○ Basal—before production of diabetes. X After beef—before production of diabetes.

made following the ingestion of glucose are not included in this figure. The correlation exhibited is statistically significant.

## DISCUSSION

Young's discovery (10) that a permanent diabetes may follow a course of injections of anterior pituitary extract has given workers a very valuable type of experimental animal. One of its many advantages is that it affords an opportunity to study the course of the disease and to correlate various observations with the intensity of the diabetes. It is from this viewpoint that much of our material is presented.

It is evident from the results shown in figure 1 that the 16 to 24-hour fasting blood glucose values in moderately or severely diabetic dogs have but little correlation with the severity of the diabetes as indicated by the proportion of the available glucose of the food excreted in the urine. This wide range of blood glucose values may possibly be related to variations in kidney function and the nutritional state of the animal as well as other uncontrolled factors. It appears in figure 1 as if a marked difference in the available glucose of the food did not have a definite effect upon the distribution of the observations. However, in dog *P16*, the only animal with sufficient material for adequate comparison, the blood glucose values during periods of meat and sugar ingestion are greater than those during the ingestion of a meat diet. Figure 1 masks this effect as it includes data from all dogs. The acute rise in blood glucose following the ingestion of meat or glucose has been reported previously (7). The high blood glucose values 16 to 24 hours after food and insulin during periods of insulin

therapy are possibly attributable to the higher liver glycogen values in the treated dogs (1). Marks and Young (2) have reported considerably higher blood glucose values than those found in our dogs, but the number of hours since the ingestion of food was not stated.

It is not surprising that the serum fatty acids are elevated in the more severely diabetic of our pituitary-diabetic dogs as similar observations have been made in human diabetes mellitus (5) and in the partially depancreatized dog (11), as well as in the totaly depancreatized dog (12); but in the latter type of diabetes the picture is complicated by the effect of the loss of some pancreatic factor other than insulin. Also during the period of injection of anterior pituitary extract an elevated value has been found (4). It is noteworthy, however, that the elevation of the serum fatty acids in our permanently diabetic dogs tended to increase with the increasing severity of the diabetes and was especially high after the diabetes became very severe. It may possibly serve as a more accurate indication of very severe diabetes than the fasting blood glucose values.

The decrease in the concentration of serum fatty acids following insulin therapy is in accord with observations upon diabetic patients (5). The failure of the fatty acids to increase following fat feeding to the dog excreting 80% of the available glucose, as it did in the dogs with total urinary diabetes, is probably attributable to the lesser intensity of the diabetes. Similar observations have been made in man (13).

Although fat feeding had no effect upon the glycosuria or ketonuria (despite apparently normal fat absorption) in the dogs with total urinary diabetes, the effect of meat feeding was very marked. The ketonuria when eating meat was considerably greater than the highest values found when fasting or receiving a fat diet even though the calculated amount of ketones that might have been formed from the fat ingested was much greater than that formed from the ingested meat. This may possibly be associated with the increased metabolism following the ingestion of meat. The disappearance or decrease of ketonuria when a fat diet is substituted for a meat diet in moderately diabetic dogs has been previously noted (2, 7). Marks and Young (2) have presented data indicating that there may be a special factor in meat other than the protein content that is responsible for its ketogenic effect.

It has been noted that the feeding of fat to dogs with total urinary diabetes did not increase the ketonuria despite elevation of the serum fatty acids, whereas persistent ketonuria did not occur in any of the dogs until the diabetes was of such a severity that there was a persistent elevation of the serum fatty acids. Thus it is probable that the concentration of serum fatty acids (within certain limits) does not have more than a slight effect upon the rate of fat catabolism. These considerations also afford further support to the concept that the elevated serum fatty acids (not attributable to the diet) are due to increased mobilization to meet metabolic requirements.

A small amount of data concerning the respiratory metabolism of pituitary-diabetic dogs has been previously presented (14, 15, 16); and Marks and Young (2) have recently published a full series of observations upon 3 pituitary-diabetic dogs. They found a 12% greater calculated heat production in their pituitary-diabetic than in normal dogs. The difference was considered to be "barely significant" statistically. No observations were recorded by these authors concerning the effect of insulin on the metabolic rate. After the production of a moderately severe diabetes in our dog *P23* there was an increase in basal oxygen consumption of 19% above that observed before the animal had been made diabetic. Furthermore, adequate insulin therapy in both dogs tested was associated with a marked reduction in oxygen consumption. These results are in accord with the observations of Hedon (17), Marks

and Young (2) and others that totally depancreatized dogs not receiving insulin have an abnormally high rate of oxygen consumption.

The average respiratory quotient of the untreated diabetic dogs was 0.02 lower than that found by Marks and Young for their pituitary-diabetic dogs, but is the same as that observed in their depancreatized dogs. These authors observed no change in the R.Q. of their animals following the ingestion of glucose. In one of our dogs there was a slight increase. The increase in the R.Q. following the administration of insulin and glucose is further evidence of the lack of inhibition of insulin action in the permanent phase of pituitary-diabetes (1).

A correlation of the oxygen consumption with the 24-hour nitrogen excretion has been observed by Wishart (18) in non-diabetic human subjects. A relationship between the oxygen consumption and the approximate nitrogen excretion at the time of the observation has been found in our diabetic dogs as shown in figure 6. It is presumably a reflection of the fact that except for a few instances they were ingesting a meat diet and utilizing but little carbohydrate.

## SUMMARY

Observations have been made upon dogs during the course of the permanent diabetes that followed a series of injections of an anterior pituitary extract. These observations have been correlated with the approximate severity of the diabetes as indicated by the proportion of the available glucose of the diet excreted in the urine.

*A. Blood glucose and serum lipids.* In dogs with moderate and severe pituitary-diabetes the concentration of the blood glucose 16 to 24 hours after the last feeding varied widely and showed but little relationship to the proportion of available glucose of the food excreted in the urine. It thus appeared to be a poor index of the severity of the diabetes at this stage.

The concentration of serum fatty acids was above normal in the more severe stages of the diabetes. This increase became particularly marked in dogs excreting over 90% of the available glucose of the diet. Persistent acetonuria did not appear until persistent elevation of the serum fatty acids was present. However, there was no correlation between the quantity of ketonuria and the concentration of the serum fatty acids in individual determinations.

Insulin therapy resulted in a decreased concentration of the elevated serum fatty acids in severely diabetic dogs.

In two dogs exhibiting total urinary diabetes the withdrawal of insulin combined with fat or meat feeding resulted in a marked increase in the concentration of serum fatty acid. The cessation of insulin treatment with fasting was associated with very little change.

In one dog excreting approximately 80% of the available glucose of the diet the feeding of fat did not result in an increased concentration of serum fatty acids.

*B. Urinary glucose, nitrogen and ketones.* In two dogs exhibiting total urinary diabetes the feeding of fat for 5 days without insulin treatment did not increase the glycosuria or ketonuria above the levels observed during similar fasting periods.

The feeding of meat to the two dogs with total urinary diabetes was associated with a total glucose and nitrogen loss greater than could be accounted for by the meat fed; and by a ketonuria much greater than during the fasting or fat feeding periods.

Comparable studies in the dog excreting about 80% of the available glucose of the diet were made. They also indicated the marked ketogenic effect of meat feedings in comparison to fat. During fat feeding or fasting very little glucose or ketones were excreted.

C. *Respiratory metabolism.* The basal oxygen consumption was significantly increased above the pre-diabetic level. The average respiratory quotient of the three untreated diabetic dogs on a meat diet was 0.68 compared to 0.73 found in the non-diabetic dog.

In diabetic animals insulin administration was associated with a reduction in oxygen consumption.

The administration of glucose to two diabetic dogs was followed by a slight increase in the average R.Q. in one dog and no change in the other. When given with insulin the R.Q. was similar to that of a normal dog given glucose alone.

Beef feeding was followed by a marked increase in oxygen consumption in all cases. In the diabetic dogs there was little or no change in the average R.Q. while in the normal dog the R.Q. approximated that of protein.

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# THE EFFECT OF REFEEDING AND OF THE ADMINISTRATION OF A PITUITARY EXTRACT ON THE OVARIES OF UNDERNOURISHED GUINEA PIGS[1]

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IN A PREVIOUS PUBLICATION (1) it was shown that undernutrition of the guinea pig resulted in marked structural changes in the thyroid, characterized by atrophy and flattening of the acinar epithelium and retention of colloid suggesting a resting, inactive gland. Refeeding resulted in a rapid gain in body weight and return of thyroid structure to normal. The thyroid of the undernourished animals was markedly sensitive to stimulation by the thyrotropic principle of the anterior hypophysis. Evidence was presented which indicated that the structural changes observed in the thyroid of the undernourished animals were due to impairment of the thyrotropic function of the anterior pituitary. The changes occurring in the ovaries of these animals are described in the present communication.

For this study mature, female guinea pigs, weighing approximately 300 gm., were selected. Details of the care and feeding of these animals have been described (1). Animals eating a full diet were killed while gaining weight, to serve as normal controls. Undernutrition was produced by feeding an inadequate diet resulting in a loss of 20 to 30% of body weight in a period of 2 weeks. In these animals, the effects of refeeding and of the administration of a pituitary extract[2] were studied. The extract was administered subcutaneously on 4 successive days; on the fifth day, the animals were killed, the ovaries removed, weighed and fixed in Zenker's solution. The organs were embedded in paraffin, sectioned and stained with hematoxylin and eosin.

The experimental conditions and their effect on the ovaries of these animals are summarized in table 1. The ovaries of the normal animals, given an adequate diet, contained numerous follicles in all stages of growth and atresia (fig. 1). Some ovaries contained normal corpora lutea. In the ovaries of all, most noticeable in those without corpora lutea, there were follicles with large antra and proliferating granulosa and theca interna, as well as other follicles of equal size showing degenerative changes in the granulosa. The average weight of the paired ovaries of 16 normal animals was 54.2 mg.

The ovaries of the undernourished animals were atrophic and showed marked changes of a retrogressive type. The average weight of paired ovaries of 19 under-

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An abstract of this paper was read by title at the annual meeting of the American Society for Clinical Investigation at Atlantic City, May 6, 1940 (*J. Clin. Invest.* 19: 784. 1940).

[2] The antuitrin-T used in this study was generously provided by Parke, Davis & Co.

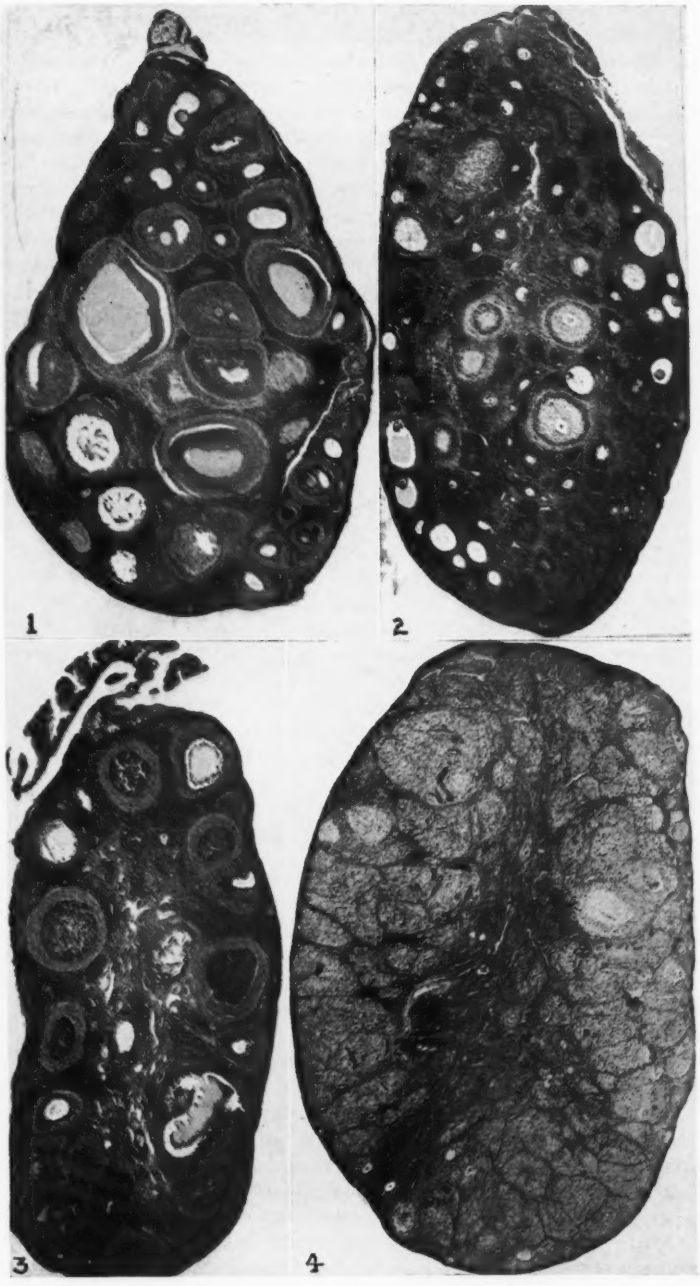


Fig. 1. The ovary of a normal guinea pig. Fig. 2. The ovary of an undernourished guinea pig Fig. 3. The ovary of an undernourished guinea pig, which had been refed a full diet and regained to above the original body weight. Fig. 4. The ovary of an undernourished guinea pig which had been given 4 daily doses of 0.01 cc. of antuitrin-T. (All photographs ×20.)

nourished animals was 26.5 mg. The most noticeable change histologically was the virtual disappearance of follicles (fig. 2). An occasional follicle with a small antrum was present but even these were considerably reduced in number. No large follicles with actively growing granulosa were seen. The interstitial tissue was relatively more conspicuous than in the normal animals, chiefly because of the disappearance of the large follicles, but the interstitial cells were all very small and the nuclei were closely packed together. The nuclei showed clumping of the chromatin at the periphery in a cartwheel formation. Similar 'cartwheel' cells have been described as a typical deficiency reaction of the ovary after hypophysectomy (2).

The addition of vitamin supplements to the inadequate diet had no effect on the

TABLE 1

| Conditions | No. of animals | Av. initial body weight | Av. final body weight | Av. weight 2 ovaries | Ovarian stimulation |
|---|---|---|---|---|---|
| | | gm. | gm. | mg. | |
| Normal Animals | 16 | — | 300 | 54.2 | — |
| Saline 0.5 cc.×4 | 5 | 294 | 300 | 50.2 | o |
| Pit. Ext. 0.0025 cc.×4 | 5 | 317 | 323 | 44.0 | ++ |
| Pit. Ext. 0.005 cc.×4 | 5 | 300 | 285 | 46.3 | +++ |
| Pit. Ext. 0.01 cc.×4 | 5 | 301 | 303 | 49.9 | +++ |
| Pit. Ext. 0.02 cc.×4 | 5 | 310 | 314 | 48.7 | +++ |
| Thyroxin 0.5 mg.×4 | 5 | 298 | 271 | 48.0 | o |
| Undernourished animals | 19 | 298 | 226 | 26.5 | — |
| Vitamins | 9 | 305 | 231 | 33.2 | o |
| Thyroxin 0.5 mg.×4 | 5 | 322 | 214 | 32.6 | o |
| Saline 0.5 cc.×4 | 5 | 314 | 231 | 29.1 | o |
| Pit. Ext. 0.0005 cc.×4 | 5 | 297 | 220 | 21.1 | o |
| Pit. Ext. 0.001 cc.×4 | 6 | 310 | 220 | 30.2 | ++ |
| Pit. Ext. 0.0025 cc.×4 | 7 | 310 | 222 | 31.5 | ++ |
| Pit. Ext. 0.005 cc.×4 | 8 | 300 | 208 | 34.4 | +++ |
| Pit. Ext. 0.01 cc.×4 | 8 | 308 | 224 | 50.6 | ++++ |
| Pit. Ext. 0.02 cc.×4 | 5 | 302 | 213 | 49.9 | ++++ |
| Refed | 7 | 310 – 226 – 323 | | 52.7 | ++ |

atrophic and retrogressive changes observed in the ovary or in the thyroid (1). Weight loss of comparable degree induced by the administration of thyroxin to animals given an unlimited diet, in a similar period of time, did not result in similar retrogressive changes in the ovaries.

A group of animals was given an inadequate diet for 2 weeks to induce undernutrition, and then given a full diet without other treatment for 2 weeks, at the end of which time they had regained to above the original body weight. The ovaries of these animals showed definite evidences of recovery and approached the normal in appearance (fig. 3). Numerous actively growing follicles with proliferating granulosa and theca interna were present. The average weight of paired ovaries of 7 such animals was 52.7 mg.

Groups of animals were given graded doses of anterior pituitary extract for 4 days as indicated in table 1. The daily administration of 0.0005 cc. of this extract was without effect on the ovaries of the undernourished animals but with amounts varying from 0.001 to 0.02 cc. daily, definite gonadotropic effect was apparent in the weight and structure of the ovaries (fig. 4). With larger doses, there was marked luteinization of the ovaries. Virtually no follicles were present but the general arrangement of the tissue, along with the finding of occasional ova in the center of masses of hyperplastic

cells, suggested that there had been marked stimulation and luteinization of all the small follicles. The cells were very large with conspicuous cytoplasm and the nuclei were large and vesicular. Occasional mitoses were present. In no instance were structures resembling normal corpora lutea to be seen. Similar histological changes were observed in the ovaries of normal animals given the larger doses of the pituitary extract.

It was thought that the ovarian stimulation may have been due, in some way, to the thyroid stimulation resulting from the thyrotropic factor. To test this possibility, a group of undernourished animals was given daily injections of 0.5 mg. of synthetic thyroxin[2] for 4 days. No evidence of stimulation was found in the ovaries of these animals.

The gonadotropic effect of this particular pituitary extract was of interest in that the ovaries show no evidences of follicular stimulation with either the intraperitoneal or subcutaneous administration. The extract was assayed by the manufacturer, and independently by us, to contain from 50 to 100 guinea pig units of thyrotropic factor per cubic centimeter. Amounts of the extract up to 0.6 cc., divided into 6 doses administered over a period of 3 days, had no effect on the weights of the ovaries of 25-day-old female rats but did result in increases in the weight of the seminal vesicles of 25-day-old male rats. As little as 0.005 cc. of the extract resulted in ovulation in sexually mature rabbits. These observations suggest that, in addition to the thyrotropic factor, this fraction contained considerable amounts of LH to account for its gonadotropic action and, at most, relatively small amounts of FSH. They also show that a pituitary extract can be prepared which has virtually no effect on the ovary of the 25-day-old rat, but at the same time may still have a powerful gonadotropic effect on the rabbit ovary. In this instance 0.6 cc. was without effect in the rat but 0.005 cc. was sufficient to induce ovulation in the sexually mature rabbit.

The rapidity with which atrophic and retrogressive changes occurred in the ovaries of the undernourished guinea pigs is worthy of emphasis. Within a period of 2 weeks, the administration of a diet of inadequate caloric content resulted in changes in the ovaries similar in character and degree to those following hypophysectomy.

The ovary of the undernourished guinea pig remains responsive to gonadotropic stimulation. The retrogressive changes which occur in the undernourished animals are fully reversible, since the administration of a full diet results in a return of normal ovarian structure. These observations are considered to support the contention that changes occurring in the ovary during inanition may be due, at least in part, to the inability of the pituitary to continue to produce sufficient gonadotropic hormone to maintain the ovaries in a functional state when the food is markedly curtailed. It should be emphasized, however, that the pituitary extract used in these experiments did not restore the ovary of the undernourished animal to normal. It only produced luteinization (or interstitial cell stimulation) and did not produce normal follicles or corpora lutea.

Similar and confirmatory observations have been made by investigators working with other animal species. In 1929 Marrian and Parkes (3) observed that partial inanition in rats resulted in ovarian atrophy and anestrus. The ovaries of their animals remained sensitive to stimulation by anterior pituitary extracts and refeeding resulted in return of estrus when body weight was regained. Selye and Collip (4), Werner (5), and Mulinos and his associates (6) have also described ovarian atrophy or anestrus in undernourished rats and have attributed them to diminished secretion of the hypophyseal gonadotropic principle. Breneman (7) has attributed the decreased testicular

[2] The thyroxin used in this study was provided by Roche-Organon, Inc.

weight of chicks on a limited diet to decreased production of pituitary gonadotropic hormones. Moore and Samuels (8) have described changes in the seminal vesicles and prostates of undernourished rats similar to those seen in castrate animals. These changes were corrected not only by the testis hormone but also by the administration of the anterior pituitary-like principle obtained from pregnancy urine, suggesting deficiency of testis hormone in the undernourished animals. Mulinos and Pomerantz (9) recently have emphasized the similarity of the histological changes in the organs of undernourished animals to those occurring after hypophysectomy and regard the state of chronic undernutrition as one of "pseudohypophysectomy." Jackson (10), and more recently Werner (5) and Mulinos and Pomerantz (9), have described atrophic and degenerative changes in the anterior pituitary of undernourished rats. Mason and Wolfe (11) and Werner (5) have found that the gonadotropic function of the hypophysis is diminished in undernourished rats.

## SUMMARY

In female guinea pigs, undernutrition of sufficient degree to cause a loss of 20 to 30% of body weight in a period of 2 weeks, results in atrophic and retrogressive changes in ovaries similar in character and degree to those following hypophysectomy. These changes are not affected by the administration of vitamin supplements but are reversible; with refeeding, normal ovarian structure is restored. The ovary of the undernourished guinea pig remains responsive to stimulation by at least one (LH) of the gonadotropic principles of the anterior hypophysis. These observations are considered to support the contention that changes occurring in the ovary during inanition may be due, at least in part, to inability of the anterior pituitary to continue to produce sufficient gonadotropic hormone to maintain the normal structure and function of the ovary when the food intake is markedly curtailed.

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# EXCRETION OF ESTROGENIC AND ANDROGENIC SUBSTANCES BY FEMALE AND MALE CHIMPANZEES WITH KNOWN MATING BEHAVIOR RECORDS[1]

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Of the investigations in which qualitative and quantitative determinations of the estrogenic and androgenic substances excreted in the urine of laboratory primates have been made, none has been inclusive to the extent that values have been ascertained for the urinary estrogens and androgens excreted throughout the cycle by the females and by the males. The only work with which the writers are familiar includes determinations of total estrogens excreted at different stages of the menstrual cycle by the female chimpanzee (1) and total estrogens and androgens in samples of urine from male (2) and female (3) *Macacus rhesus*.

The result was that a comparison of the estrogens and androgens excreted by an infrahuman primate with those excreted by man was not possible. It was apparent that the monkey differs from man in that the levels of urinary estrogens and androgens are low in both sexes and that in the female chimpanzee the level of excreted estrogens is nearer that found in women. It was not known, however, whether or not the quantity of androgens excreted by the female chimpanzee also approaches that excreted by the male chimpanzee. Finally, Allen, Diddle, Burford and Elder (1) showed that a peak of estrogen excretion occurs about the middle of the cycle in the female chimpanzee, but their work does not exclude the possibility that a second peak might occur shortly before menstruation as in man (4–9).

## MATERIALS AND METHODS

Twenty-three-hour urine specimens were collected from 2 adult female and 4 adult male chimpanzees. The age of the 2 females, *Mamo* and *Nira*, is estimated to be 12 and 15 years, respectively. That of the 4 males, *Jack*, *Pan*, *Bokar* and *Frank*, is estimated to be 19, 18, 15 and 10 years, respectively (10).

*Mamo* had twice been subjected to hysterotomy and on the second occasion, May 20, 1939, her right ovary was removed. Since then menstruation, with two exceptions, has been regular at intervals of 28 to 37 days. There was no evidence of its occurrence in October and December, 1939, although tumescence and detumescence were normal. For a half hour each day from April 6 to May 24, 1940, the door between her cage and that occupied by a vasectomized male, *Pan*, was left open. During this half hour when the animals had access to each other, they were observed

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for mating activity, the direction of movement with respect to each other, the time spent in grooming and playing, and indications of timidity, self-confidence, hostility or friendliness. During both periods of maximal swelling which occurred while these observations were in progress, *Mamo* presented for copulation. During the first cycle presentation occurred daily from the 6th through the 18th day with the exception of the 14th day. Copulation occurred from the 8th through the 17th day with the exception of the 14th day. During the second cycle presentation occurred from the 10th through the 18th day and copulation from the 13th through the 18th day. At the beginning of the periods of observation *Mamo* took the initiative in joining *Pan* 47 out of 48 times. During the observations she left him to enter the other cage 199 times, but she joined him 198 times. The freedom with which she moved back and forth is interpreted as reflecting an absence of fear. Frequently the animals played together, the initiative being taken sometimes by one animal and sometimes by the other. The periods of play were not correlated with any stage of the menstrual cycle. *Mamo's* behavior during the period of observation, her condition when urine was being collected and records for the past 10 years indicate that she is a healthy, normal animal.

*Nira* is a normal appearing, intact female who has menstruated at intervals of 33 to 46 days since February, 1936, when a mild illness was reported. Her outstanding peculiarity is her consistent avoidance of sexual contacts with males, even at the time of maximal swelling. For a half hour each day from April 12 to May 23, 1940, a normal male, *Jack*, was given access to her, and data comparable with those obtained for *Mamo* were collected. One period of sexual swelling occurred, but not once did she present for copulation. In contrast to *Mamo*, she did not take the initiative in joining the male when the door between the cages was opened. The male came to her 41 of 42 times. The predominant direction of movement was away from the male. She left him 146 times and joined him only 29 times. Much of her movement was clearly an expression of timidity and fear although on no occasion did the male seem inclined to attack her or to be aggressive. No behavior was displayed which could be characterized as play.

The urine voided during 23-hour periods was collected from each animal on alternate days, beginning June 2 and ending July 6, 1940, in the case of *Mamo*, and beginning May 30 and ending July 9 in the case of *Nira*. The urine collected July 7 from *Nira* was lost during the extraction process. Collection from *Mamo* was begun the 29th day of the cycle which was 6 days after the completion of detumescence and 2 days before the beginning of menstruation. The last collection was made on the first day of the succeeding menstruation. Collection from *Nira* was begun the 15th day of the cycle during the period of maximal swelling. The last specimen was collected the first day of detumescence in the succeeding cycle.

The urine flowed over the floor of the urine collecting cage and was filtered as it passed through cotton into a collecting bottle. The first thing in the morning and at frequent intervals during the daylight hours it was transferred to a bottle kept in the refrigerator and which contained 3 cc. of toluene as a preservative. The animals were moved from the urine collecting cage each morning at 8 and between 8 and 9 the cage and collecting bottle and funnel were cleaned thoroughly. Immediately prior to shipment to New Haven for extraction and assay, 10 cc. of concentrated hydrochloric acid were added for each 100 cc. of urine.

The general features of behavior observed during the weeks immediately prior to the time the urine was collected did not differ from what is known about the behavior during the previous years' confinement in the colony. It is assumed, therefore, that

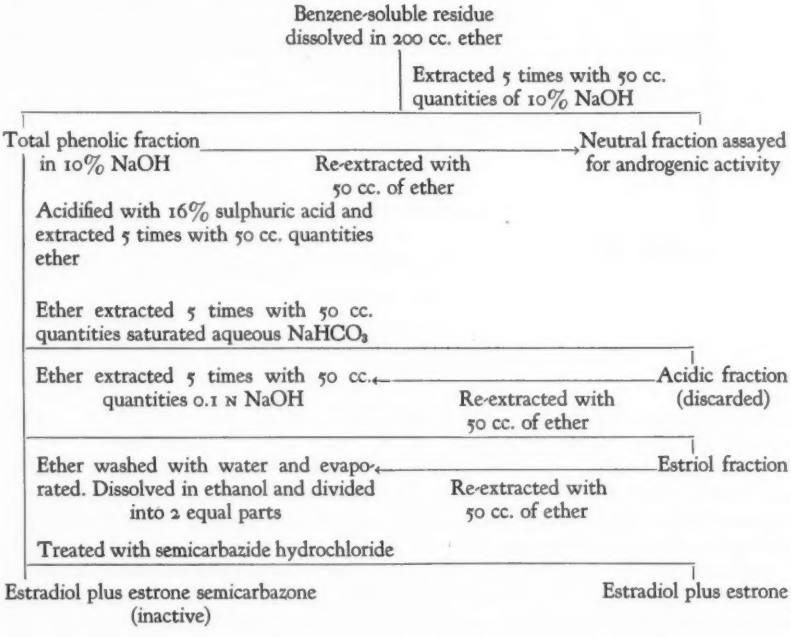
had observations been made while urine was being collected, no important differences would have been shown.

Three 23-hour specimens of urine were collected from each of the 4 males during the period from July 16 to July 28, 1940. *Pan* was sterilized by ligature and resection of the ducti deferentes April 13, 1937. Otherwise the animals are normal. The sexual interest of *Pan*, *Bokar* and *Jack* is vigorous. In the past *Frank* has been more or less indifferent even in the presence of receptive females. In every other respect he is a normal, vigorous male.

The routine of urine collection was similar to that followed when collections were being made from the females.

Hydrolysis and extraction of the urine were carried out simultaneously by a modification of the method of Dingemanse, Borchardt and Laqueur (11). Each acidified urine sample was refluxed for 6 to 8 hours with 250 cc. of benzene. After cooling the benzene was decanted. Two hundred fifty cc. of fresh benzene were added and decanted. The procedure was repeated once more. The benzene solutions were then combined and evaporated in vacuo.

The benzene extracts of the specimens from the females were treated according to the outline given below.


Benzene-soluble residue dissolved in 200 cc. ether
Extracted 5 times with 50 cc. quantities of 10% NaOH
Total phenolic fraction in 10% NaOH
Re-extracted with 50 cc. of ether
Neutral fraction assayed for androgenic activity
Acidified with 16% sulphuric acid and extracted 5 times with 50 cc. quantities ether
Ether extracted 5 times with 50 cc. quantities saturated aqueous NaHCO3
Ether extracted 5 times with 50 cc. quantities 0.1 N NaOH
Re-extracted with 50 cc. of ether
Acidic fraction (discarded)
Ether washed with water and evaporated. Dissolved in ethanol and divided into 2 equal parts
Re-extracted with 50 cc. of ether
Estriol fraction
Treated with semicarbazide hydrochloride
Estradiol plus estrone semicarbazone (inactive)
Estradiol plus estrone

It was observed that by separating the acidic compounds from the ether solution containing the total phenolic fraction far more material passed into the bicarbonate solution than when this operation was performed on the ether solution of the total benzene-soluble residue.

When the method of purification used in this investigation is employed, the estrogens are fractionated to permit the estimation of estradiol, estrone and estriol.

These fractions do not necessarily contain only these compounds because other unknown estrogens may be found with them. For convenience the fractions will be referred to simply as estradiol, estrone and estriol.

The method used for the estimation of both estrone and estradiol in the 10% alkali-soluble fraction depends on the inactivation of the ketone by semicarbazone formation. To 10 cc. of ethanol containing one-half the 10% alkali-soluble compounds were added 100 mg. of semicarbazide hydrochloride and 100 mg. of sodium acetate. The mixture was refluxed 4 hours, cooled, dissolved in ether and washed with water. The residue, after evaporation of the ether, was dried by distilling absolute ethanol from it in vacuo. By subtracting the value of the estrogenic activity of the semicarbazide treated portion (estradiol plus inactivated estrone) from the value of the

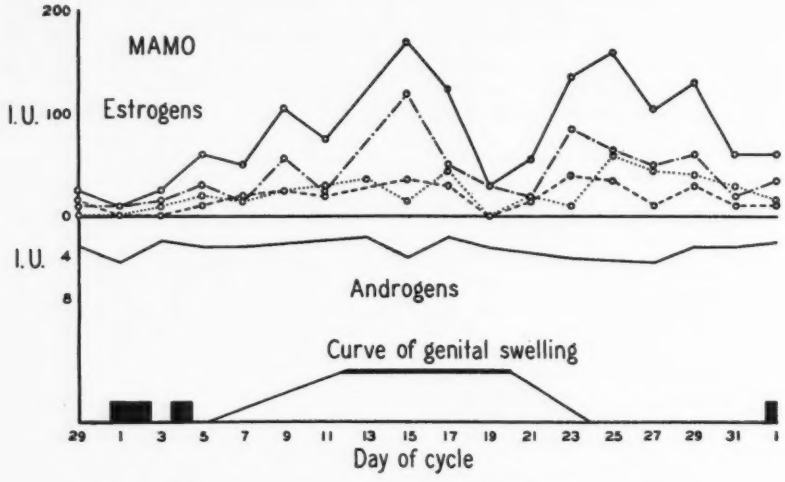



Fig. 1. Estrogen and androgen excretion by female chimpanzee, *Mamo*. All values are expressed in I.U. per day. Same key as in figure 2.

untreated portion (estrone plus estradiol), the quantity of estrone in the 10% alkali-soluble fraction was estimated.

The extracts of the specimens from the males were separated into total phenolic and neutral fractions only. As with the female specimens, the acidic compounds were separated from the phenolic substances after rather than before the separation of the latter from the neutral compounds.

The spayed, mature female mouse was employed in the assays of the estrogens. Proestrous, estrous and metestrous smears were considered positive. For each assay at least 6 and usually 10 or more animals were used. Six animals were used only when limited amounts of material were available. All values for estrogenic activity are expressed in International Units (I.U.), the activity of 0.1 microgram of estrone.

The androgenic substances were assayed on day-old chicks in accordance with the method of Dorfman and Greulich (12). At least 6 and usually 10 chicks were used for each assay. The values for androgenic substances are recorded in terms of International Units, the activity of 0.1 mg. of androsterone.

In order to facilitate comparison with data previously obtained from other species, all values for both estrogens and androgens are recorded on a 24-hour basis.

EXPERIMENTAL

*Estrogen excretion by female chimpanzees.* The values for estrogens excreted by the female chimpanzees are given above the abscissae in figures 1 and 2. Because the limit of sensitivity of the bioassay method is 10 I.U., all values less than this are recorded as zero. The sexual condition is indicated by the curve of genital swelling at the bottom of each graph and the days of the menstrual cycle are counted from the first day of the preceding menstruation.

(*a*) *Levels of estrogens excreted by Mamo* (fig. 1). The amount of total estrogens varied from 10 I.U. per 24 hours during the first menstruation to 170 I.U. at the time of maximal genital swelling. Following a gradual rise which started during menstruation, maximal excretion occurred on the 15th day of the cycle. The peak was followed by a rapid decrease in the quantity excreted until the 19th day, when another low

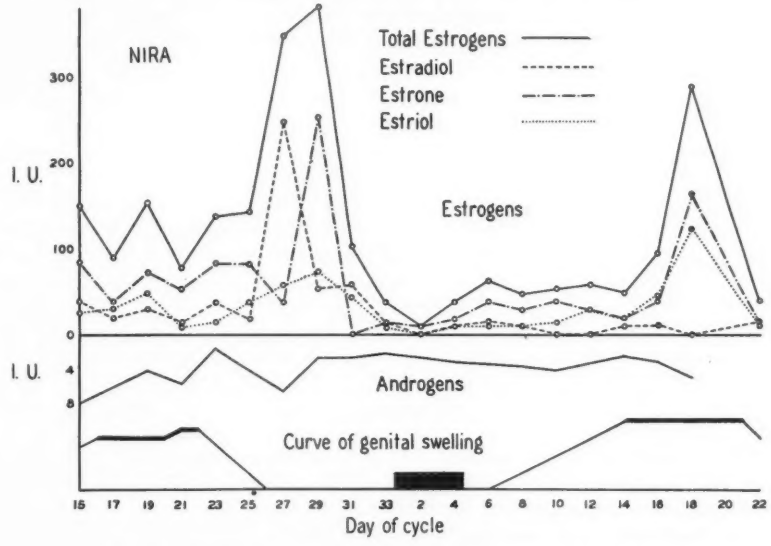



Fig. 2. ESTROGEN AND ANDROGEN EXCRETION BY FEMALE CHIMPANZEE, *Nira*. Menstruation is represented by solid area on lower base line.

point was reached. Even at this time detumescence had not begun. It was first observable 1 or 2 days later when the amount of excreted estrogen was rising toward the premenstrual peak which was reached on the 25th day. A second sharp decrease began between the 29th and 31st days, and the next menstruation followed on the 33rd day.

The levels of estradiol, estrone and estriol followed the same general trend throughout the cycle. Estrone excretion exceeded that of estradiol and estriol throughout most of the cycle; on the first day of menstruation and on the 19th day estrone alone was detected.

(*b*) *Levels of estrogens excreted by Nira* (fig. 2). The collection of urine from *Nira* was started on the 15th day of the cycle. Therefore, the values given in figure 2 represent the second half of one menstrual cycle (*A*) and the first half of the succeeding cycle (*B*). The range of values of total estrogens, from 10 I.U. during menstruation to

385 I.U. during the premenstrual period in cycle *A*, was considerably greater than that found for *Mamo*. Nevertheless, most of the relationships were similar. The quantity of total estrogens was lowest during menstruation and rose to peaks during the premenstrual period in cycle *A* and at the time of maximal swelling in cycle *B*. In contrast to the relationships in *Mamo*, the peak prior to menstruation is higher than that during maximal swelling.

The trend of excretion of the three components is different from that shown by *Mamo* chiefly in that the estrone values are lower relative to estradiol and estriol and in the greater proportion of excreted estradiol during the premenstrual period in cycle *A*.

*Androgen excretion by female chimpanzees.* The values for the androgenic activity of the female urine are given below the abscissae in figures 1 and 2. Until more data

TABLE 1. ANDROGEN AND ESTROGEN EXCRETION BY MALE CHIMPANZEES

| Animal | Date of collection | Urine volume | Androgenic activity | Estrogenic activity |
|---|---|---|---|---|
| | | cc. | I.U. | I.U. |
| *Jack* | July 19 | 500 | 4 | 35 |
| | July 23 | 500 | 4 | 85 |
| | July 27 | 800 | 5 | 65 |
| *Pan* | July 18 | 1040 | 6 | 50 |
| | July 22 | 875 | 6 | 135 |
| | July 28 | 730 | 12.5 | 40 |
| *Bokar* | July 16 | 1000 | 6 | 55 |
| | July 20 | 1170 | 3 | 40 |
| | July 26 | 1220 | 8.5 | 40 |
| *Frank* | July 17 | 890 | 7.5 | 30 |
| | July 21 | 930 | 6 | 125 |
| | July 25 | 1340 | 13.5 | 135 |
| | Average | | 6.8 | 70 |

can be obtained, no significance should be attached to the irregular variations from 2 to 4.5 I.U., average 3.1 I.U., in the specimens from *Mamo*, and from 1.5 to 8 I.U., average 3.7 I.U., in the specimens from *Nira*.

*Androgen and estrogen excretion by male chimpanzees.* The quantities of androgens and estrogens excreted by the 4 male chimpanzees are shown in table 1. The levels vary somewhat from animal to animal. The quantity of androgens which averages 6.8 I.U. is twice that found in the urine from the 2 females who excreted an average of 3.4 I.U. per day. The quantity of estrogens which averages 70 I.U. per day is somewhat less than the average of 105 I.U. excreted by *Mamo* and *Nira*.

## DISCUSSION

The results of the present investigation confirm the conclusions of Allen, Diddle, Burford and Elder (1) to the extent that high values of estrogenic substances are obtained when the genital swelling of the chimpanzee is maximal, and that low values are obtained during menstruation. Beyond this, however, several refinements and additions, which are of interest, can be made.

One of the most important is the discovery that in the female chimpanzee, as in the human female, a second peak of estrogen excretion occurs shortly before menstruation. As in man, the significance of this peak is not understood although it may be associated with the activity of the corpus luteum which is functional at this time.

It is clear, however, that the degree of sexual swelling does not parallel the amount of estrogenic substances excreted except shortly before the middle of the cycle. Moreover, the willingness to copulate is not necessarily associated with the excretion of large quantities of estrogens. During the premenstrual period, when the amount of excreted estrogens is large, presentation and mating are infrequent (13, 14).

The relatively long interval between the first decrease in estrogen excretion and the beginning of detumescence does not seem to have been noted heretofore. It may represent a latent period between the time when factors responsible for detumescence first appear and the time when their effect is manifested externally. The point can be elucidated only when replacement therapies, which can be accompanied by observation of genital changes and bioassays, are possible.

As far as the excretion of estrogens is concerned, the chimpanzee appears to occupy a position intermediate between man and the Rhesus monkey. The amount of estrogen excreted by adult women, 24 to 34 years of age, varies from 50 to 600 I.U. per day, average 270 I.U. (15, 16). The 2 female chimpanzees from which data were obtained excreted 10 to 385 I.U. per day, average 105 I.U. By contrast, an average of only 10 I.U. has been found in specimens from female monkeys (3). The quantity of estrogens excreted by the human male varies from 20 to 290 I.U. per day, average 100 I.U. (15, 16). That excreted by the male chimpanzees varied from 30 to 135 I.U., average 70 I.U. per day, and that excreted by male monkeys amounted to only 1.1 to 2.5 I.U. per day (2).

With respect to the excretion of androgens also, the chimpanzee occupies an intermediate position but it appears to be much nearer the monkey than man. The human female, 24 to 34 years of age, excretes 30 to 100 I.U. per day, average 47 I.U. (15, 16). The female chimpanzee, on the other hand, excretes an average of only 3.4 I.U. per day, and the female Rhesus monkey nearly as much or an average of 2.3 I.U. per day (3). The relationship between these species with respect to the quantity of androgens excreted by the males is not different. Human males, 25 to 35 years of age, excrete 40 to 100 I.U. per day, average 66 I.U. (15, 16), but the male chimpanzee excretes an average of only 6.8 I.U. per day, and the male monkey an average of 2.6 I.U. per day (2).

The lack of relationship between the quantity of excreted estrogen and sexual behavior as it is seen in the cycles of *Mamo* and *Nira* is noted elsewhere (p. 589). Entirely different in its nature is the problem presented by such an animal as *Nira* in which there is every indication of normal hormonal output but an obvious failure of response in sexual relationships. When allowance is made for the phylogenetic position of the chimpanzee one is tempted to ascribe *Nira's* behavior to unanalyzed social or environmental factors. However, the likelihood of somatic or constitutional factors cannot be dismissed without further study. Guinea pigs and rats have been found which gave evidence of being normal except for their persistent refusal to mate (17, 18, 19). After they were spayed, copulatory responses were induced by the injection of larger than normal quantities of estrogen followed by normal amounts of progesterone. Therefore, the refractoriness was attributed to a low sensitivity to the conditioning action of estrogen. That the explanation of *Nira's* failure to mate will be found to be as simple as this is doubted. Nevertheless, until proof is given that social or environmental factors are involved, the possibility of the existence of an abnormally high threshold to hormonal stimulation should not summarily be rejected.

## SUMMARY

Bioassays have been made of estrogenic and androgenic substances found in 23-hour urine samples from 2 adult female chimpanzees throughout the menstrual cycle and from 4 adult males. One of the females consistently avoids sexual contacts with

the males although the cyclic structural changes associated with reproduction appear to be normal. In the females, peaks of estrogen excretion coincided with maximal genital swelling and with the premenstrual period. Until more data can be obtained, no significance should be attached to the irregular variations in androgens excreted by the females. The males excreted twice as much androgenic substances as the females and somewhat less estrogenic substances. The quantity of estrogenic substances excreted by female and male chimpanzees was intermediate between that excreted by man and the Rhesus monkey. The quantity of androgenic substances excreted by chimpanzees of both sexes was nearer that excreted by the monkey. No relationship was found between the sexual behavior of the animals and the quantity of excreted estrogenic or androgenic substances.

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# PERCUTANEOUS ADMINISTRATION OF ESTROGENS FOLLOWED BY PROGESTIN IN INDUCING SEXUAL RECEPTIVITY IN SPAYED GUINEA PIGS[1]

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IN A PREVIOUS PUBLICATION (1) we reported that diethylstilbestrol, the synthetic estrogen discovered by Dodds and his coworkers (2), when followed by the subcutaneous administration of progestin, would induce sexual receptivity in spayed guinea pigs. The results obtained both by injection and percutaneous administration of this synthetic estrogen were comparable with those obtained when equal doses of estrone were used. Since then we have extended this work to include the percutaneous administration of progestin, as well as the estrogen, the thought being that perhaps in this way we could obtain some information of the effectiveness of progestin by this route of administration.

Since the first report of Zondek (3) in 1938, on the cutaneous application of follicular hormone, as well as progestin, several papers have appeared dealing with this mode of administration for estrogens and male hormones (4–7) but as far as the authors are aware, no other reports on progestin have appeared. Zondek (3, 5) reported that alcohol was a better medium for the application of the sex hormones than oil. With respect to progesterone he merely showed that larger doses were required for percutaneous administration than for subcutaneous injection in young rabbits.

## EXPERIMENTAL

The spayed guinea pigs used in this work were the same animals referred to in the previous publication (1). As in this previous work, both the estrone and the stilbestrol were dissolved in cottonseed oil and rubbed on the shaved necks of the animals. The oil solutions were rubbed on 4 times over a period of 2 days, and the progestin was rubbed on during the morning of the third day. The total amount of oil solution containing the estrogen rubbed on each animal was 0.1 cc.

In some of the experiments the progestin was dissolved in cottonseed oil, and rubbed on the shaved necks of the animals, while in other experiments it was dissolved in a mixture composed of 15% glycerol and 85% ethyl alcohol. In all cases the total volume of solution rubbed on each animal was 0.02 cc. The results are summarized in the tables.

## RESULTS AND DISCUSSION

The latent period and the receptive period shown in the table are those defined by Dempsey and coworkers (8, 9). The latent period is the time between the administration of the progestin, which in this work is the percutaneous application, and the first definite response of receptivity; and the receptive period is the time the animal will respond when stroked in the dorso-lumbar region.

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TABLE 1. EFFECT OF PERCUTANEOUS ADMINISTRATION OF ESTROGEN IN OIL FOLLOWED BY PERCUTANEOUS ADMINISTRATION OF PROGESTIN IN OIL IN CAUSING HEAT IN SPAYED GUINEA PIGS

| Total amt. of estrogen rubbed on | Amt. of progestin rubbed on in oil | Number treated | Number receptive period | Mean latent | Mean length of receptive period | Combined latent and receptive periods |
|---|---|---|---|---|---|---|
| γ | I.U. | | | *hr.* | *hr.* | *hr.* |
| Estrone | | | | | | |
| 10 | 0.20 | 10 | 8 | 4.7 | 10.0 | 14.7 |
| 10 | 0.10 | 9 | 6 | 5.9 | 4.1 | 10.0 |
| 10 | 0.05 | 10 | 5 | 9.6 | 4.1[1] | |
| 10 | 0.03 | 12 | 5 | 10.0 | 4.5[1] | |
| Stilbestrol | | | | | | |
| 10 | 0.40 | 4 | 4 | 4.9 | 7.4 | 12.3 |
| 10 | 0.30 | 4 | 4 | 5.9 | 6.5[1] | |
| 10 | 0.20 | 10 | 0 | — | — | |
| 10 | 0.10 | 10 | 0 | — | — | |
| 15 | 0.20 | 10 | 5 | 6.4 | 6.9 | 13.3 |
| 15 | 0.10 | 10 | 1 | 9.5 | 4.0 | 13.5 |
| 15 | 0.05 | 10 | 0 | — | — | — |

[1] Animals still receptive.

Inspection of tables 1 and 2 shows that progestin is effective in this reaction when applied percutaneously either in oil or in an alcohol-glycerol mixture. The latent period and the receptive period in both cases compare favorably with what was obtained when progestin was injected subcutaneously. It can be seen, however, that more progestin was required when it was rubbed on the animal than when it was injected. This is brought out clearly in table 3, which is a summary giving a direct comparison of the various modes of administration of the progestin. The values shown in this table are averages of several tests conducted at different times. Occasionally considerable variation has been experienced from test to test. This table shows clearly the fact that when the amount of estrogen rubbed on is held constant, more progestin is required percutaneously than by injection to obtain comparable effects.

One can obtain a rough approximation of this difference in response by noting the amount of progestin required under the various situations to give a 50% response. Thus when estrone was used as the estrogen, it required 2 to 3 times more progestin

TABLE 2. EFFECT OF PERCUTANEOUS ADMINISTRATION OF ESTROGEN IN OIL FOLLOWED BY PERCUTANEOUS ADMINISTRATION OF PROGESTIN IN ALCOHOL-GLYCEROL SOLUTION IN CAUSING HEAT IN SPAYED GUINEA PIGS

| Total amt. of estrogen rubbed on | Amt. of progestin rubbed on in alcohol-glycerol sol. | Number treated | Number receptive | Mean latent period | Mean length of receptive period | Combined latent and receptive periods |
|---|---|---|---|---|---|---|
| γ | I.U. | | | *hr.* | *hr.* | *hr.* |
| Estrone | | | | | | |
| 10 | 0.20 | 8 | 8 | 6.1 | 7.1[1] | |
| 10 | 0.10 | 8 | 7 | 7.5 | 5.7[1] | |
| 10 | 0.05 | 8 | 3 | 7.2 | 4.8[1] | |
| 10 | 0.03 | 8 | 0 | | | |
| Stilbestrol | | | | | | |
| 10 | 0.20 | 10 | 3 | 6.4 | 3.8 | 10.2 |
| 10 | 0.10 | 10 | 0 | | | |
| 10 | 0.05 | 10 | 0 | | | |
| 15 | 0.20 | 9 | 9 | 7.6 | 6.0[1] | |
| 15 | 0.10 | 9 | 5 | 7.0 | 5.0 | 12.0 |
| 15 | 0.05 | 10 | 0 | | | |

[1] Animals still receptive.

when rubbed on in oil or in alcohol solution than when it was injected in oil to get the same effect. With stilbestrol as the estrogen, it required 6 to 8 times as much progestin by rubbing on as by injection.

Moreover, these results show that estrone, weight for weight, is about two times as effective as stilbestrol in this test. When the amount of stilbestrol administered is increased from 10 γ to 15 γ, there is a similar increase in responsiveness of the animals, even though the amounts of progestin on each dose level are the same as those used

TABLE 3. COMPARISON OF THE PERCUTANEOUS AND SUBCUTANEOUS METHODS OF ADMINISTRATION OF PROGESTIN IN CAUSING HEAT IN SPAYED GUINEA PIGS CONDITIONED WITH ESTROGEN

| Total amt. of estrogen rubbed on | Progestin injected in oil | % receptive | Progestin rubbed on in oil | % receptive | Progestin rubbed on in alcohol-glycerol | % receptive |
|---|---|---|---|---|---|---|
| γ | I.U. | | I.U. | | I.U. | |
| Estrone | | | | | | |
| 10 | | | 0.20 | 80 | 0.20 | 100 |
| 10 | 0.10 | 90 | 0.10 | 70 | 0.10 | 90 |
| 10 | 0.05 | 90 | 0.05 | 50 | 0.05 | 40 |
| 10 | 0.03 | 90 | 0.03 | 40 | 0.03 | 0 |
| 10 | 0.02 | 70 | | | | |
| 10 | 0.01 | 20 | | | | |
| Stilbestrol | | | | | | |
| 10 | | | 0.40 | 100 | | |
| 10 | | | 0.30 | 100 | | |
| 10 | | | 0.20 | 0 | 0.20 | 30 |
| 10 | 0.10 | 100 | 0.10 | 0 | 0.10 | 0 |
| 10 | 0.05 | 70 | | | 0.05 | 0 |
| 10 | 0.03 | 50 | | | | |
| 10 | 0.02 | 10 | | | | |
| 10 | 0.01 | 0 | | | | |
| 15 | | | 0.20 | 50 | 0.20 | 100 |
| 15 | | | 0.10 | 10 | 0.10 | 60 |
| 15 | | | 0.05 | 0 | 0.05 | 0 |

for the 10 γ dose. This shows the mutual coordination between the action of estrogen and progestin in causing this response.

There appears to be very little difference between the oil and the alcohol-glycerol mixture as solvents for the progestin when applied percutaneously. It might appear from the results shown in table 3 that the alcohol-glycerol mixture was a better solvent than oil for the progestin when used on animals conditioned with stilbestrol. Considerably more tests would have to be carried out to prove this point.

Since the first publication of this series (1) we have extended the administration of progestin by injection in oil to lower levels as shown in columns 1, 2, and 3 of table 3.

It can be noted that this affords a fairly rapid and simple method of determining semi-quantitatively small amounts of progestin. Moreover, the test animals can be used repeatedly without any ill effects. As can be seen from table 3, a 20% response is obtained with 0.01 I.U. progestin and a 70% response with 0.02 I.U. when 10 γ of estrone is used as the conditioning agent. If at least 10 animals per dose are used, one can determine this difference in response rather easily.

## SUMMARY

Sexual receptivity was induced in spayed guinea pigs by estrone plus progestin, and by stilbestrol plus progestin, when the substances were applied percutaneously. Estrone was about twice as effective in this respect as stilbestrol.

The estrogens were administered in oil, while progestin was dissolved in an alcohol-glycerol solution in some experiments and in oil in others with only a slight difference in results.

When 10 γ of estrone was used as the conditioning estrogen, it required 2 to 3 times more progestin when rubbed on in oil or in alcohol solution than when it was injected in oil to get the same effect. With 10 γ of stilbestrol as the conditioning estrogen, it required 6 to 8 times as much progestin by rubbing on as by injection.

These results are of interest because they suggest the possibility of the use of the percutaneous method of administration of progestin in clinical cases, and thus avoiding the present rather unsatisfactory use of injections of oils.

A fairly rapid and simple method of determining semi-quantitatively small amounts of progestin has been presented.

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# ENDOCRINE CONTROL OF THE LIPID METABOLISM OF THE BIRD. IV. LIPID METABOLISM OF THE BIRD DURING PUBESCENCE AND THE ANNUAL REST[1]

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PREVIOUS WORK has shown that the lipid metabolism of the bird is influenced by the ovarian estrogenic hormone (1–3). The rapidity of the response of the blood lipids to estrogen injections suggests that the blood lipid level may offer a good method for determining changes in ovarian activity within this animal. In the present investigation, lipid levels have been correlated with ovarian activity during 2 periods in the life of the bird, pubescence and the annual rest, for it is in these periods that the most rapid and pronounced changes in ovaries occur.

## EXPERIMENTAL

Single comb White Leghorn chickens of the Poultry Division stock were employed. The birds used in the study of puberty were maintained in individual cages. At suitable intervals, as judged by comb growth, blood was removed by heart puncture, and immediately thereafter these animals were killed for examination of reproductive tract and liver. The birds used in the study of annual rest were removed from the Poultry Division flock at a time when routine autopsy reports were being made. In all cases blood and liver samples were taken for analyses 16 to 20 hours after the birds had been deprived of food. The methods employed for determination of lipids in blood and liver have been described elsewhere (4, 5). Free cholesterol was determined in an acetone solution after the phospholipids had been precipitated.

### *The Pubescent Bird*

Puberty in the bird has been defined as the interval between the onset of accelerated comb growth and the laying of the first egg (1). Although the data previously recorded were insufficient to delimit sharply the time of onset of increases in blood lipids during puberty, it was nevertheless established that the blood lipids are elevated when the interval before laying is 10 days or less (1). In the present study the concentration of lipids has been correlated with the growth and development of oviduct and ovary. Observations made on 30 birds are recorded in tables 1 and 2. The former contains the data on 16 birds in which the ova measured less than 0.5 cm. in diameter; at this stage in the development of the ovary, the deposition of yellow yolk in the ovum has not yet begun (6). Table 2 contains the data on birds whose ovaries contained ova measuring 0.7 cm. or more in diameter but in which no ovulation had yet occurred. In these ova, deposition of yellow yolk had already occurred.

The 16 birds of table 1 showed evidence of increased ovarian activity. Thus the height of the smallest comb in the birds measured 2.6 cm. as compared with an average height of 1.3 cm. in 27 prepubescent 4-month-old pullets. The mean value of the oviduct weight was 5.7 gm., the minimum 0.8 gm.; the maximum weight found for this

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TABLE 1. PUBESCENT BIRDS WITH OVARIES CONTAINING OVA MEASURING LESS THAN 0.5 CM. AND CONTAINING NO YELLOW YOLK

| | Bird weight | Comb height[2] | Reproductive tract | | Liver | | Blood lipids mg. per 100 cc. whole blood | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Ovary | Oviduct | Weight | Total fatty acids | Total cholesterol | Total fatty acids | Phospholipids | Total lipids |
| | kg. | cm. | gm. | gm. | gm. | % | | | | |
| Minimum | 1.03 | 2.6 | 0.5 | 0.8 | 16.4 | 3.41 | 74 | 181 | 198 | 258 |
| Maximum | 1.66 | 5.1 | 0.9 | 17.2 | 33.7 | 35.40 | 146 | 600 | 389 | 725 |
| Mean | 1.30 | 4.2 | 0.7 | 5.7 | 23.5 | 10.82 | 112 | 339 | 299 | 451 |
| $S.E_m$[1] | | ±0.2 | ±0.03 | ±1.0 | | ±3.01 | ±6 | ±29 | ±14 | ±34 |

(Minimum, maximum and mean for 16 birds.)

[1] Standard error of the mean.

[2] The averge comb height of 27 prepubescent birds was 1.27±0.05 cm.

TABLE 2. BLOOD LIPIDS OF PUBESCENT BIRDS

| Bird No. | Bird weight | Comb height | Reproductive tract | | | Liver | | Blood lipids mg. per 100 cc. whole blood | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Ovary weight | Ova maximum diameter | Oviduct weight | Weight | Total fatty acids | Total cholesterol | Total fatty acids | Phospholipids | Total lipids |
| | kg. | cm. | gm. | cm. | gm. | gm. | % | | | | |
| 14 | 1.14 | 3.6 | 0.5 | 0.7 | 7.2 | 16.0 | 5.19 | 97 | 374 | 342 | 471 |
| 32 | 1.32 | 5.1 | 2.9 | 1.7 | 15.4 | 40.8 | 5.77 | 117 | 617 | 402 | 734 |
| 29 | 1.46 | 5.0 | 3.5 | 1.7 | 15.9 | 31.1 | 8.40 | 149 | 813 | 505 | 962 |
| 31 | 1.54 | 4.6 | 0.9 | 0.6 | 20.6 | 37.0 | 4.48 | 296 | 3385 | 1290 | 3681 |
| 26 | 1.41 | 5.2 | 5.3 | 1.9 | 22.0 | 32.3 | 10.50 | 245 | 2673 | 1061 | 2918 |
| 21 | 1.42 | 6.5 | 2.5 | 1.3 | 22.5 | 51.8 | 28.70 | 236 | 2618 | 996 | 2854 |
| 37 | 1.38 | 5.2 | 2.1 | 1.2 | 22.8 | 32.9 | 19.10 | 244 | 2200 | 1152 | 2444 |
| 24 | 1.27 | 4.8 | 8.6 | 1.9 | 24.5 | 27.1 | 13.20 | 146 | 1860 | 753 | 2006 |
| 4 | 1.30 | 4.5 | 2.8 | 1.5 | 24.5 | 29.7 | 19.60 | 139 | 811 | 524 | 950 |
| 5 | 1.39 | 4.5 | 0.8 | 0.7 | 24.6 | 27.1 | 7.52 | 139 | 696 | 479 | 835 |
| 13 | 1.35 | 4.3 | 14.5 | 2.5 | 29.8 | 27.1 | 6.44 | 182 | 2682 | 1008 | 2864 |
| 15 | 1.25 | 4.3 | 12.7 | 2.5 | 30.3 | 35.2 | 19.80 | 151 | 1472 | 688 | 1623 |
| 20 | 1.38 | 6.3 | 15.6 | 2.4 | 31.8 | 28.6 | 7.63 | 154 | 1450 | 680 | 1604 |
| 30 | 1.23 | 4.5 | 29.2 | 2.3 | 32.6 | 28.3 | 6.79 | 343 | 5112 | 1849 | 5455 |
| 22 | 1.36 | 5.5 | 20.1 | 2.9 | 37.4 | 30.5 | 5.57 | 177 | 1368 | 835 | 1545 |

These birds contained ovaries with ova that measured more than 0.5 cm. in diameter and in which yellow yolk had been deposited. Ovulation had not yet occurred.

TABLE 3. BIRDS APPROACHING REST WITH ACTIVE OR SEMI-ACTIVE OVARIES

| Bird No. | Bird age | Bird weight | Laying history | | | Molting history[1] | Reproductive tract | | | | Blood lipids mg. per 100 cc. whole blood | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Age at first egg | Total eggs laid | Interval since last egg laid | | Ovary weight | Oviduct weight | No. of yolks (active) | Observations[2] | Cholesterol | | | Total fatty acids | Phospholipids | Total lipid |
| | | | | | | | | | | | Total | Free | Ester | | | |
| | days | kg. | days | | days | | gm. | gm. | | | | | | | | |
| P-1097 | 545 | 1.52 | 190 | 118 | 4 | 2 | 31 | 56 | 4 | P R | 98 | 44 | 54 | 770 | 358 | 868 |
| P-755 | 531 | 1.62 | 157 | 171 | 4 | 3 | 47 | 55 | 5 | P R | 90 | 64 | 26 | 654 | 446 | 744 |
| P-39 | 559 | 1.89 | 150 | 144 | 1 | — | 37 | 50 | 4 | | 181 | 158 | 23 | 2722 | 511 | 2903 |
| P-130 | 559 | 1.54 | 187 | 170 | 1 | — | 33 | 52 | 4 | | 120 | 100 | 20 | 1140 | 625 | 1260 |
| P-1083 | 545 | 1.86 | 193 | 177 | 2 | — | 59 | 55 | 4 | | 71 | 71 | 0 | 658 | 560 | 729 |
| P-734 | 531 | 1.53 | 192 | 167 | 3 | 3 | 32 | 43 | 4 | | 78 | 63 | 15 | 532 | 352 | 610 |

[1] The birds were examined for evidence of molt by Mr. H. B. Mugglestone, Division of Poultry Husbandry, University of California at Berkeley, on three occasions: (1) July 7, (2) August 22 and (3) October 1. The numbers in the table refer to the examination on which molting was first observed. None of the birds recorded in tables 3–5 were reported to have completed their molt at the time of the third examination. A minus sign (—) indicates the bird had not yet started to molt.

[2] P indicates the presence of a partially formed egg in the oviduct; D, the presence of degenerating yolks in the ovary in addition to the actively growing yolks noted in the preceding column. R indicates partially resorbed egg material in the abdominal cavity.

structure in the prepubescent immature bird was 0.25 gm. Despite these signs of increased ovarian activity, the mean values as well as their variability (as measured by the standard error of the means) of all lipid constituents were fairly similar to those previously recorded for the immature bird (1). In only 2 birds was the level of total fatty acids somewhat greater than the maximum value previously found for the immature bird, namely 494 mg. per 100 cc. of whole blood. One of these contained an oviduct weighing 8.7 gm., and the total fatty acid content of its blood was 600; in the other an oviduct weighing 17.2 gm. was found, and its blood contained 532 mg. of total fatty acids. These oviducts were 2 of the 3 largest observed in the entire group.

The height of the combs of birds with actively growing ova (table 2) varied from 3.6 to 6.5 cm. Their oviducts weighed from 7.2 to 37.4 gm. With one exception, elevated lipid levels were present in these animals. The highest value for total lipid was 5455 mg., and this reflected rises in cholesterol, phospholipid and fatty acids. Although yellow yolk was present in the ova of all 15 birds, the deposition of yellow yolk need not be associated with a blood lipid rise. Thus *bird 14* contained ova measuring 0.7 cm. in diameter, but the level of its blood lipids had not risen above that found in the immature bird.

*The relation of oviduct size to the level of the blood lipids in the pubescent bird.* During growth of the oviduct, no significant change in the lipid level of the blood occurred in birds in which oviduct growth up to a weight of approximately 9 gm. had been attained. The highest value for total fatty acids previously found for immature birds was 494 mg. (1), whereas the highest value found in the present study in birds containing oviducts weighing between 0.8 and 9.0 gm. (tables 1 and 2) was 600 mg. The values for cholesterol and for phospholipids in the birds with oviducts weighing 0.8 to 9.0 gm. were essentially similar to those observed in the immature female bird.

The remainder of the birds dealt with in table 2 contained oviducts weighing between 15.4 and 37.4 gm., and in these the lipid level of the blood was well above that observed in the immature state. The values for total lipids fluctuated between 734 and 5455 mg. per 100 cc. of whole blood. As in the case of the laying bird (1), the various lipid constituents did not respond equally, the most pronounced rises occurring in phospholipids and fatty acids. Changes were not observed in the cholesterol level of the whole blood until very marked increases had already appeared in phospholipid and fatty acids. Thus, despite the fact that in *bird 24* total fatty acids had reached a value of 1860 mg. and phospholipid 753 mg.—as compared with highest values of 600 and 389 mg. respectively in the earlier series recorded above—the cholesterol level of the blood of this animal showed no striking rise. Pronounced increases in cholesterol did occur, however, but only in those birds in which total fatty acid values were found above 2000 mg. per 100 cc. of whole blood.

Although an elevation in the blood lipid level was found when the oviduct attained a size of 15 gm. or over, no exact correlation was observed between the rise of any of the lipid constituents and the size of the oviduct. This is particularly well shown in birds 30 and 22, in which values for blood total fatty acids were 5112 and 1368 mg. and corresponding weights of oviducts were 32.6 and 37.4 gm. Again, birds 24, 4 and 5 had oviducts weighing approximately 25 gm., yet considerable differences were noted in the levels of total fatty acids, phospholipids and total lipid.

*Liver lipids of the pubescent bird.* The results shown in table 1 clearly establish that an increase in the fatty acid content of the liver may precede the rise in blood lipids. It should be noted, however, that a number of birds in puberty as well as in lay (5) have livers with no greater lipid content than those observed in the immature birds (5).

In the birds recorded in table 1, values for total fatty acids as high as 35.4% were observed at a time when no significant change had yet occurred in the lipid level of the blood. In pubescent birds the fatty acid content of the liver bears no apparent re-

TABLE 4. BIRDS IN REST WITH DORMANT OVARIES[1]

| | Bird age | Bird weight | Laying history | | | Reproductive tract[2] | | Blood lipids mg. per 100 cc. whole blood | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Age at first egg | Total eggs laid | Interval since last egg | Ovary weight | Oviduct weight | Cholesterol | | | Total fatty acids | Phospholipids | Total lipids |
| | | | | | | | | Total | Free | Ester | | | |
| | *days* | *kg.* | *days* | | *days* | *gm.* | *gm.* | | | | | | |
| Minimum | 531 | 1.09 | 144 | 27 | 15 | 2 | 2 | 77 | 49 | 8 | 216 | 177 | 312 |
| Maximum | 559 | 2.20 | 280 | 200 | 135 | 8 | 20 | 204 | 106 | 115 | 615 | 444 | 722 |
| Mean | 544 | 1.48 | 199 | 127 | 56 | 3.4 | 7.6 | 123 | 75 | 48 | 362 | 315 | 485 |
| $S.E_m$ | | | | | | ±0.3 | ±1.0 | ±7 | ±3 | ±6 | ±19 | ±15 | ±24 |

(Minimum, maximum and mean values for 22 birds.)
[1] Molting history as follows (see footnote table 3): 2 birds, (1); 8 birds, (2); 9 birds, (3); 3 birds (−).
[2] Observations as follows (see footnote table 3): 1 bird, (D), 11 birds, (R).

lation to oviduct size or ovary weight or the maximum diameter of the ovum, for an increased fatty acid content of the liver may be present irrespective of size of these structures. The variation in the fatty acid content of the liver is worthy of note. Thus in 14 birds, which showed a growth of the oviduct from 0.8 to 8.8 gm., the values of liver fatty acids fluctuated from 3.4 to 35.4% (table 1). The bird with the smallest oviduct, namely 0.8 gm., had 13.65% fatty acids in its liver. The highest fatty acid content in the liver (35.40%) was found in a bird that had an oviduct weighing only 4.0 gm., whereas the bird with the largest oviduct, namely 17.2 gm., had only 4.17% fatty acids in its liver. In birds in which growth of the oviduct between 15.4 and 37.4 gm. had been attained (table 2) liver fatty acids fluctuated from 4.48 to 28.70, but again these fluctuations were without relation to the development of either ovary or oviduct.

### *The Annual Rest*

Birds hatched between February and April usually begin to lay between August and November of their first year of life. Laying continues for approximately 9 to 14 months and is usually followed by an interval during which a complete cessation of laying occurs. This latter period, termed here for convenience the annual rest, includes the period of molt. It is of variable duration. Some birds do not stop production and hence never enter the annual rest. Others may remain out of production 6 months or more. In view of the interesting observations already made on the bird during the actively laying state and during the period approaching this state, namely pubescence, it seemed highly desirable to extend these lipid studies to the annual rest and to the

TABLE 5. BIRDS COMING OUT OF REST WITH ACTIVE OVARIES

| Bird No. | Bird age | Bird weight | Laying history | | | Molting history[1] | Reproductive tract | | | | Blood lipids mg. per 100 cc. whole blood | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Age at first egg | Total eggs laid | Interval since last egg laid | | Ovary weight | Oviduct weight | No. of yolks (active) | Observations[2] | Cholesterol | | | Total fatty acids | Phospholipids | Total lipids |
| | | | | | | | | | | | Total | Free | Ester | | | |
| | *days* | *kg.* | *days* | | *days* | | *gm.* | *gm.* | | | | | | | | |
| P-718 | 538 | 1.60 | 205 | 135 | 16 | 2 | 3 | 9 | 1 | | 94 | 56 | 38 | 358 | 445 | 452 |
| P-1258 | 545 | 1.47 | 180 | 156 | 52 | 3 | 9 | 13 | 1 | D | 142 | 89 | 53 | 735 | 435 | 877 |
| P-930 | 559 | 1.76 | 196 | 171 | 60 | 3 | 12 | 15 | 1 | D | 183 | 105 | 78 | 740 | 420 | 923 |
| P-418 | 545 | 1.95 | 162 | 107 | 66 | 3 | 8 | 23 | 2 | | 111 | 77 | 34 | 312 | 324 | 423 |
| P-656 | 531 | 1.40 | 146 | 160 | 121 | 2 | 8 | 38 | 2 | | 118 | 94 | 24 | 652 | 371 | 770 |
| P-501 | 538 | 1.35 | 143 | 128 | 85 | 2 | 33 | 47 | 3 | R | 96 | 77 | 19 | 879 | 545 | 975 |
| P-649 | 531 | 1.81 | 145 | 31 | 41 | — | 32 | 48 | 3 | R | 120 | 89 | 31 | 1232 | 529 | 1352 |
| P-1310 | 545 | 1.90 | 173 | 126 | 33 | 3 | 36 | 49 | 4 | R | 70 | 48 | 22 | 690 | 479 | 760 |
| P-1208 | 538 | 1.72 | 182 | 191 | 89 | 3 | 24 | 51 | 4 | DR | 65 | 63 | 2 | 312 | 305 | 377 |
| P-1100 | 545 | 1.91 | 138 | 62 | 123 | — | 38 | 51 | 4 | R | 105 | 81 | 24 | 1131 | 528 | 1236 |
| P-1139 | 548 | 1.74 | 165 | 158 | 131 | — | 54 | 59 | 4 | R | 175 | 138 | 37 | 2340 | 840 | 2515 |
| P-712 | 538 | 0.72 | 165 | 131 | 46 | 3 | 50 | 64 | 4 | D | 140 | 112 | 28 | 1480 | 660 | 1620 |

[1] See footnote table 3. [2] See footnote table 3.

intervals immediately preceding and following the annual rest, periods in which birds are coming into and out of lay. Forty birds in all were studied; the lipid levels of their blood are recorded in tables 3, 4 and 5. These birds were selected at random from a group removed from the flock at the end of the laying year. This latter group, however, had not been selected at random; it included only birds that showed either a low-to-average record of egg production or an abnormal record.

Blood samples were obtained on a single day, October 2, for 40 trap-nested birds, after which they were immediately killed for examination of the reproductive tract. Birds with inactive ovaries were designated as being in the annual rest. Birds with active ovaries (i.e. ovaries containing actively growing yolks) were classified on the basis of their egg production into 1) approaching rest (table 3), or 2) coming out of rest (table 5). Observations on the condition of the oviduct and ovary as well as the presence or absence of degenerating ova also aided in the classification. The condition of the feathers was found to be a less reliable guide, since some birds go out of production long before the molt starts and others continue to lay throughout the molting period.

*The pre-rest period.* Birds classified as approaching rest are recorded in table 3. The ovaries of these birds still contained actively growing yolks, and all had laid within the 4 days preceding the observations. The oviducts of 2 birds contained partially formed eggs. Three birds (*P-734*, *P-755* and *P-1097*) had already begun to molt. *P-1097* had been molting for over 40 days and very likely would have gone through the winter without stopping its egg production.

The lipid values of these birds were characteristic of those previously reported for laying birds (1). The values for total lipid fluctuated between 610 and 2903 mg. Not all lipid constituents share in the elevated lipid level. Phospholipids were increased in a number of cases, but the most significant rises occurred in total fatty acids.

*Birds in annual rest.* The birds recorded in table 4 were out of lay for periods varying from 15 to 135 days at the time they were killed for study. No active yolks were observed in them. The highest value found for total blood lipids in the 22 birds shown in table 4 was 722 mg.; in all cases the levels of cholesterol (both free and esterified), phospholipid and fatty acids approximated those previously recorded for the male and immature female bird (1). Indeed, the close correspondence between the blood lipid values recorded in table 4 and those previously observed in immature birds (1), with respect to means and variability, is striking.

*Birds coming out of annual rest.* The birds recorded in table 5 were designated as coming out of annual rest by the presence of actively growing yolks in the ovaries and by the fact that they had laid no eggs for some time. With a single exception (*P-718*), these birds had been out of egg production for at least one month. *P-718* had rested for 4 months, had then laid 4 eggs and again stopped laying during the 16 days before she was killed. Her oviduct weighed only 9 gm., but the presence of a single actively growing yolk suggested the onset of renewed ovarian activity.

Nine of the 12 birds had an increased concentration of blood lipids, and in 4 cases the elevation in blood lipid was quite marked. Thus total lipids in *P-1139* *P-1100*, *P-712* and *P-649* were respectively 2515, 1236, 1620 and 1352 mg. Cholesterol failed to show much response, and the rises in total lipids reflected changes mainly in fatty acids and, to a much lesser extent, phospholipid.

It should be noted that the classification "coming out of rest" is none too definite. For example, 3 birds (*P-649*, *P-1100* and *P-1139*) which had been out of production for 41 to 131 days had not yet began to molt. These 3 birds had abdominal egg matter and hence should probably be looked upon as abdominal layers that had not yet come into rest. In many birds a few ovulated yolks are retained in the abdomen. For unknown reasons, a few birds retain all yolks and yet continue to ovulate for some time.

Some, at least, of the other birds with abdominal egg matter might also have had active ovaries during the period in which they were out of production.

*The relation of the blood lipid level to oviduct size in birds in the annual rest, approaching rest and emerging from rest.* It was noted in the case of the pubescent bird that an elevation of blood lipids occurred when an oviduct weight of 15 gm. or more had been attained. The data obtained from birds in the annual rest as well as from birds approaching and emerging from rest lend further support to this relation between oviduct size and elevation in blood lipids.

The oviducts of the laying birds weigh about 40 gm. In birds in annual rest the oviducts are no longer the massive structures observed in the laying state, and, as noted above, the level of the blood lipids is similar to that found in the male or the immature bird. In all but 3 (*P-1062*, *P-1217* and *P-408*) of the 22 birds from which the summarized data in table 4 were derived, the oviducts weighed less than 15 gm. In birds *P-1062* and *P-1217* the oviduct weighed 16 gm., whereas in *P-408* it weighed 20 gm. These oviducts were probably still regressing. Thus *P-1062* had been out of production for only 18 days. Although *P-408* had been out of production for 30 days, nevertheless the presence of abdominal egg matter suggests that her ovary may have been inactive for a much shorter time.

In birds approaching rest (table 3) the elevated lipid levels of the blood were associated with oviducts weighing 43 to 56 gm.

Ten of the 12 birds emerging from rest (table 5) and in possession of oviducts weighing 15 gm. or more showed elevated lipid levels in their blood. Bird *P-1208* was exceptional in that it had an oviduct weighing 51 gm. despite a blood lipid level characteristic of the immature state. The presence of degenerating ova in this bird suggests that a recent depression in ovarian activity had again occurred.

It is evident that a correlation exists between the size of the oviduct and the levels of the blood lipid constituents. The lack of a more exact relationship, however, can best be explained by the fact that the blood lipids can fluctuate more rapidly in response to estrogen levels than the oviduct can. In other words, the size of the oviduct at a given time is the result of the action of estrogen over a period of several days, whereas the blood lipids reflect the levels of estrogen during the past several hours.

## SUMMARY

The lipid metabolism of the bird was investigated during the periods of its life when the most rapid changes in ovarian activity are occurring: pubescence, and the approach of the annual rest, the rest itself and emergence from it.

The blood lipid level of pubescent birds increases abruptly about the time the oviduct has grown to a weight of 15 to 18 gm. No close relation exists between the onset of yellow yolk deposition in the developing ovum and the blood lipid level.

Fatty livers occur in the pubescent bird. Increases in the lipid content of the liver may precede increases in the lipid levels of the blood.

Elevated lipid levels are usually present in the bird until the onset of the annual rest, at which time the blood lipids return to values approximating those found in the immature state. Increased lipid levels are again present in the blood of birds emerging from rest.

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# THE EFFECTS OF PREGNENINOLONE AND RELATED STEROIDS ON SEXUAL DEVELOPMENT IN FISH (*Lebistes reticulatus*)

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THE AVAILABILITY of steroid substances in crystalline form, similar or identical to those produced in the gonads and adrenal cortex, has given great impetus to investigations of their possible roles in vertebrate sexual determination and differentiation.

The effects of crystalline sex hormones, in fish, on secondary sex characters and gonads have been studied by several investigators (1–8) but effects of substances chemically related to these hormones have not been investigated except in the case of ovipositor-lengthening in the bitterling (9, 10).

Pregneninolone,[1] a synthetic steroid prepared and described by Inhoffen and associates (11, 11a) possesses progestational activity per os in mammals. It also has the properties of an estrogen and is weakly androgenic in birds (12). This compound, as far as we are aware, has not previously been administered to any vertebrates lower than birds.

This work, a continuation of previous studies with testosterone propionate (7), was undertaken with the purpose of observing the effects of pregneninolone and other synthetic and naturally occurring steroids on the development of secondary sex characters and gonads of both sexes of the fish, *Lebistes reticulatus*.

Six different steroid preparations were used, namely: pregneninolone, testosterone propionate, progesterone, desoxycorticosterone acetate, pregnandiol, and adrenal cortical extract.

## METHODS

Both treated and control fish were fed a basic diet of tropical fish food (Blue Ribbon) supplemented with *Daphnia*, *Tubifex*, and brine shrimp (*Artemia*). The water was thermostatically controlled at approximately 78° F.

A total of 70 fish were fed pregneninolone tablets. These were freely eaten when crumbled into the aquaria. Four groups of animals (51 fish) were treated from birth for periods varying from 14 days to 56 days. Two of these groups (23 fish) were each given approximately 10 mg. of pregneninolone per week; another group (16 fish), 5 mg. per week, and the fourth group (12 fish), a total of 5 mg. which was added to the aquarium at birth. Two more groups consisting of 9 mature males and 10 mature females were each given 10 mg. of pregneninolone per week over a period of 44 days and 50 days respectively. Twenty animals were raised, handled, and their tissues sectioned in a manner identical with the immature treated fish except that pregneninolone treatment was not employed. Weighed and sectioned gonads of 12 normal mature males and 9 normal mature females served as controls for the mature experimental fish.

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[1] The author is indebted to Dr. Max Gilbert of the Schering Corporation for supplying testosterone propionate (oreton), desoxycorticosterone acetate (cortate), progesterone (proluton) and pregneninolone (pranone).

Twenty-one fish were injected with testosterone propionate, 15 of which were each injected with 0.025 mg. of hormone in oil of sesame when two weeks of age and weekly injections continued thereafter for 8 weeks, being gradually increased to 0.25 mg. Two mature males and 6 mature females were injected weekly with 0.1 mg. of testosterone propionate over a period of 6 weeks.

Thirty-six immature guppies were injected with progesterone, 32 with desoxycorticosterone acetate, and 13 with adrenal cortical extract in a manner somewhat similar to the testosterone treated animals. Furthermore, progesterone, desoxycorticosterone acetate, cortical extract and pregnandiol were injected separately into groups of mature males (2 in each group) and mature females (6 in each group) in weekly amounts of approximately 0.1 mg. for a period of 7 weeks.

As controls 42 litter mates were segregated at birth and raised in a manner identical with the experimental animals except that the steroid solvent, sesame oil, was injected.

The lengths and weights of most animals were measured at 42 days of age, a period in normal development just previous to external sex differentiation and divergence in size of the two sexes, and again when killed.

The dimorphic secondary sex characters studied were size, shape, coloration, sex behavior and condition of the anal fin.

After clearing in xylol, infiltrating and embedding in tissue mat, all sections were cut at 8 m$\mu$ and stained with Delafield's hemotoxylin and counterstained in eosin.

## OBSERVATIONS

*A. Effects of pregneninolone on secondary sex characters.* From its reported effects in higher vertebrates we expected pregneninolone to have a feminizing influence. Its effects were, however, purely androgenic. None of the 51 animals treated from birth showed female secondary sex characters during the period of treatment. All treated fish, regardless of age, sex and dosage, exhibited a transformation of the anal fin into a male gonopod. In fish treated from birth the modification was visible within 5 days and as the precocious development proceeded the anal fin became very elongate and extended almost to the caudal fin (fig. 2B, compare with fig. 1C). Further development into a complete gonopod was rapid when compared to normal development, requiring 20 days in fish receiving high dosages (5 to 10 mg. in tank per week) and 30 days in fish receiving low dosages (total of 5 mg. in tank). The gonopod forms in the normal male at 42 to 60 days of age.

Mature females required a longer period of treatment (10 days) before the anal fin became noticeably modified. The transformation was almost complete after 40 days of treatment (fig. 2A, compare with fig. 1A, 1B).

Most fish receiving treatment from birth, regardless of sex and dosage, exhibited masculine yellow, orange and red color patterns within 15 to 20 days after treatment began. In normal development these colors develop 55 to 65 days after birth in males only. Immature males, on the whole, were more responsive than females in these color changes but in most cases the sexes were indistinguishable and the gonads had to be studied histologically before sex could be definitely established.

In normal females, after 30 to 35 days of age, there is evident an area of black pigment at the base of the anal fin known as the 'gravid spot.' This spot was completely suppressed in all females receiving treatment from birth but in the mature female it could not be made to disappear completely.

Pregneninolone had an inhibitory effect on somatic growth of all immature fish and also completely stopped growth in growing sexually mature females. The growth of immature fish was inhibited to such an extent that 42-day-old fish were, on the average, 25% shorter than control fish of the same age.

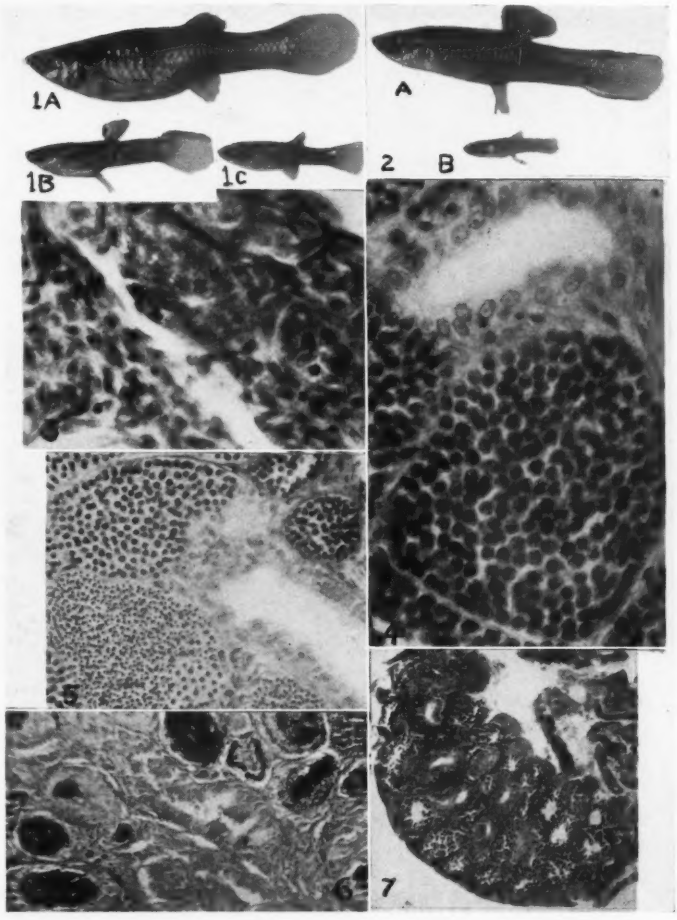


Fig. 1A. Normal mature female exhibiting typical grayish body color, unmodified anal fin and characteristic body form. ×1⅓. B. Normal mature male showing characteristic body shape, color patterns and a gonopod. ×1⅓. C. Normal immature fish lacking external sexual differentiation. ×1⅓. Fig. 2A. Female treated 30 days with pregneninolone, showing a well formed gonopod, color patterns and male body shape. ×1⅓. B. Immature fish treated from birth for 15 days with pregneninolone showing an induced gonopod. ×1⅓. Compare with fig. 1 C. Fig. 3. Cross section of a portion of a normal 14-day-old immature testis showing spermatogonia and little organization. ×375. Fig. 4. Cross section of a portion of a testis of a 14-day-old animal, treated with pregneninolone, showing a precocious cyst of primary spermatocytes and well-formed ducts. ×375. Compare with fig. 3. Fig. 5. Cross section of a portion of a testis of a 20-day-old animal, treated with pregneninolone, showing cysts of primary and secondary spermatocytes and well developed ducts. ×375. Compare with figs. 3 and 4. Fig. 6. Cross section of a portion of a testis of a 36-day-old animal, treated with pregneninolone, showing spermatocytes, spermatids, mature sperm and greatly hypertrophied testicular ducts. ×125. Compare with fig. 7. Fig. 7. Section of a normal 36-day-old testis showing primary spermatocytes and small well developed ducts. ×125.

All treated fish assumed a male body shape in that the fins were carried more closely to the body and the rotund female shape was lacking (fig. 2A, compare with fig. 1A).

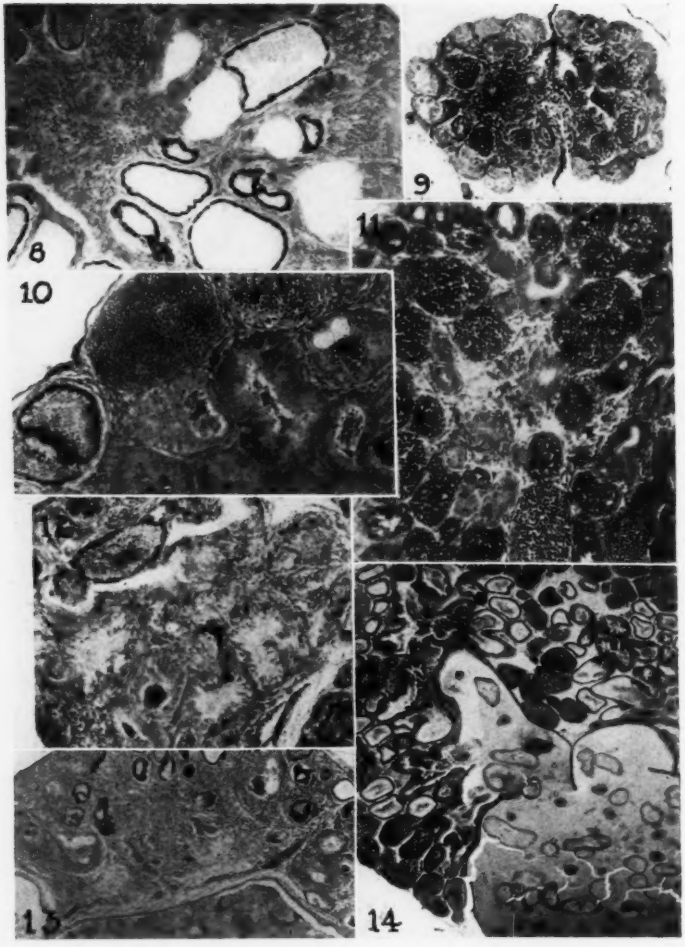


Fig. 8. Section of a testis of a 42-day-old fish, treated with low dosages of pregneninolone, showing an essentially normal, miniature, mature testis. All stages of spermatogenesis are present but later stages predominate. ×125. Compare with fig. 9, 10. Fig.9. Cross section of a normal 42-day-old testis showing peripheral spermatogonia, central spermatocytes and well defined ducts. ×125. Fig. 10. Cross section of a portion of a testis of a 42-day-old fish, treated with high dosages of pregneninolone, showing a few spermatocytes, a spermatophore, and a few abortive spermatids within hypertrophied testicular ducts. ×125. Fig. 11. Section of a 50-day-old normal testis showing spermatocytes, a number of well defined ducts, and a fairly well developed interstitium. ×125. Fig. 12. Section of a testis of a 50-day-old fish, treated with pregneninolone, showing an abnormal gonad remnant composed of duct vestiges and a few sperm cells. ×125. Compare with fig. 11. Fig. 13. Cross section of a testis of a mature fish, treated with pregneninolone for 44 days, showing an abnormal abundance of terminal spermatogenic stages, and hyperplastic areas of gonad stroma. ×50. Compare with fig. 14. Fig. 14. Cross section of an untreated mature testis, showing all stages of spermatogenesis. ×50.

Pregneninolone treatment induced male mating behavior in mature females after 20 days of treatment and also brought out this characteristic precociously in all immature fish within the same length of time.

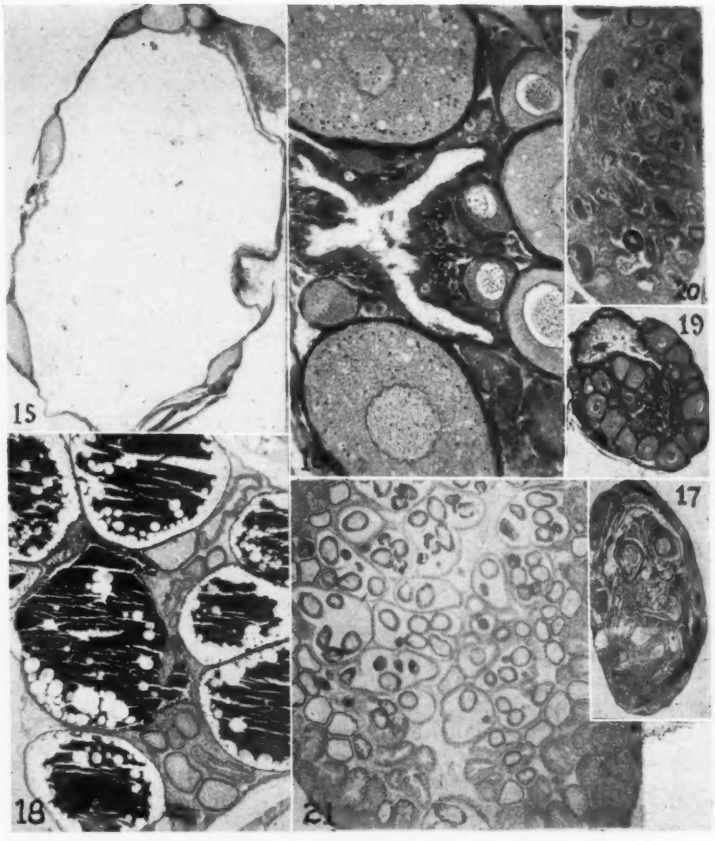


Fig. 15. Cross section of an ovary of a 50-day-old animal, treated with pregneninolone, showing a few small ovocytes in a thin layer of tissue which forms the wall of the ovarian cavity. ×45. Compare with fig. 16. Fig. 16. Cross section of a 50-day-old normal ovary, showing large ovocytes in the process of yolk deposition and an ovarian cavity lined with columnar epithelium. × 325. Fig. 17. Cross section of an ovary of an adult fish, treated with pregneninolone for 50 days, showing focal areas of degeneration around remaining strands of embryos, and a few peripheral ovocytes and ducts. ×25. Compare with fig. 18 and 19. Fig. 18. Cross section of an untreated mature ovary, showing large yolk eggs, ovocytes, and ovarian ducts. ×16. Fig. 19. Cross section of an ovary of an adult fish, treated with pregneninolone for 50 days, showing small size and ovocytes lacking yolk. ×25. Fig. 20. Section of a testis of an animal, treated with testosterone propionate for 65 days from 2 weeks of age. Note the small size, abundance of gonad stroma and absence of early spermatogenic stages. ×50. Compare with fig. 21. Fig. 21. Cross section of an untreated mature testis showing all stages of spermatogenesis. ×50.

*B. Effects of pregneninolone on testes of young males.* When treatment was started at birth there was a marked initial stimulation of the testes as indicated by a precocious maturation of the male germ cells.

Fourteen-day-old fish subjected to a total of 20 mg. of pregneninolone from birth had small but well organized testes which were composed of an abundance of precocious primary spermatocytes as well as rather large testicular ducts. The control testes were not as advanced in development—only peripheral spermatogonia being present (compare fig. 3 with fig. 4). The testes of 20-day-old treated males contained

well developed testicular ducts, primary spermatocytes and secondary spermatocytes (fig. 5). Spermatogonia were relatively scarce as compared with the control testis which contained only spermatogonia and showed no duct formation.

The testes of males treated for 36 days (35 mg. in tank) contained spermatocytes and spermatids together with an occasional group of mature sperm cells in the process of aligning themselves in spermatophore arrangement. The cells of the ducts were hypertrophied to such an extent that in some cases the lumina were obliterated. The 36-day-old control testes were much smaller than treated ones and contained spermatogonia, an abundance of primary spermatocytes and fairly well developed ducts (compare fig. 6 and 7).

Testes of fish treated for 42 days (35 mg. in tank) showed all stages of spermatogenesis but later stages were more abundant than early ones. The center of the testis was composed of intertubular, hyperplastic areas of connective tissue and great numbers of hypertrophied testicular ducts in which were found abortive spermatids and somewhat atypical spermatophores. Untreated control gonads contained an abundance of spermatogonia, primary spermatocytes, some intertubular tissue, and well developed ducts. The gonads of males receiving small dosages of pregneninolone (total of 5 mg. in tank) were not as abnormal as those receiving high dosages. The testes were small but contained all stages of spermatogenesis including mature sperm in typical spermatophore arrangement. However, late stages of spermatogenesis were more abundant than early ones (fig. 8, compare with fig. 9, 10).

Animals treated for 50 days (55 mg. in tank) contained testes which were extremely abnormal, being largely denuded of germ cells. In some, a few terminal stages of spermatogenesis were present in certain regions but near the center only an occasional atypical spermatophore could be seen. Hyperplastic areas of gonad stroma were present but most of the gonad was composed of highly ramifying duct remnants whose epithelium was in the process of dismemberment. Control testes contained primary spermatocytes, a few secondary spermatocytes, a number of well defined ducts, a fairly well developed interstitium; all of which were surrounded by a peripheral layer of nests of spermatogonia (fig. 11, compare with fig. 12).

In summary, pregneninolone induced a precocious maturation of existing germ cell elements which were hurriedly forced into the excurrent ducts in a disorganized fashion. The gonad was left exhausted and degenerate.

C. *Effects of pregneninolone on mature testes.* The testes of all treated mature males showed a divergence from the normal. The gonads were large and difficult to remove in the intact state due to the escape of gonad elements (probably sperm) from the cut end of the main testicular duct. The average gonad weight of these fish exceeded that of controls by 2 mg., a difference of probable statistical significance. The testes were lacking in spermatogonia and only a few cysts of spermatocytes were present. There was, in all cases, an unusual abundance of terminal spermatogenic stages and mature sperm. Hyperplastic areas of gonad stroma were present throughout all regions of the gonad and filled in the wide spaces between spermatophores and ducts (compare with normal mature testis, fig. 13, 14). The effects of treatment were, therefore, essentially the same as obtained in the younger animals, i.e., a hastened maturation of spermatogonia without their replacement.

D. *Effects of pregneninolone on the ovaries.* The immature female gonad was not as responsive to experimental manipulation as was the male gonad. There was no indication of sex reversal but prolonged treatment (50 days from birth) resulted in an inhibition of ovogenesis and yolk deposition and an abnormal accumulation of fluid within the ovarian cavity (fig. 15, compare with fig. 16).

Ovaries of treated mature females showed a return to a juvenile condition and if yolk and embryos were present when treatment began the gonad showed degenera-

tive changes due to resorption of these elements (fig. 17, compare with fig. 18, 19).

*E. Effects of testosterone propionate.* This work with testosterone propionate was supplementary to that previously reported (7) and will not be fully described. This substance exerted masculinizing effects on the secondary sex characters similar to those of pregneninolone with one interesting exception. It exerted an inhibitory rather than a stimulating effect on the development of red, orange and yellow color patterns—a surprising fact since these color patterns are one of the most definite male secondary sex characters. In this respect its effects were identical to those of estrogens (2).

In no dose which we have been able to give either by injection or orally has testosterone propionate exerted its androgenic action as quickly or completely as pregneninolone.

The testes of testosterone treated animals were small and could not be accurately dissected for weighing. These gonads were composed of hypertrophied testicular ducts containing spermatophores and a few abortive spermatids. Very few early stages of spermatogenesis were present but there was an abundance of hyperplastic areas of gonad stroma (fig. 20, compare with fig. 21). These effects were in general similar to those of pregneninolone.

Ovaries of testosterone treated females showed an inhibitory action apparently identical with that of pregneninolone.

*F. Steroids lacking any effect.* Progesterone, desoxycorticosterone acetate, adrenal cortical extract and pregnandiol had no effect upon sexual development in Lebistes. The treated animals, as compared with control fish injected with sesame oil, showed no difference in growth rate, times of appearance of different secondary sex characters, or in the development of gonads.

## DISCUSSION

The work with testosterone and pregneninolone reported here confirm the interpretations previously presented (7) concerning control of secondary sex characters in Lebistes.

The masculinizing effect of pregneninolone and testosterone on the anal fin is certainly the most sensitive and quickest androgenic response observed in this species. It could possibly be standardized for assay purposes.

An unusual effect of testosterone and especially pregneninolone, since no such action occurs with androgens in mammals (13) was their suppression of growth. Furthermore, estrogens stimulate growth and cause males to approach the female size (2).

The mating behavior of males would seem to be entirely dependent upon the hormone or hormones produced by the testis. In normal development testis maturity coincided with the appearance of mating behavior and this activity can be produced precociously in all fish of either sex with testosterone or pregneninolone.

The androgenic action of pregneninolone was surprisingly not shared by the closely related substances, progesterone and desoxycorticosterone acetate. Testosterone propionate does not fulfill all the expectations of an androgen in Lebistes whereas pregneninolone does. Testosterone propionate is probably less effective in inducing gonopod formation and male size, and unlike pregneninolone has an estrogen-like effect in that it inhibits the development of male red color patterns. This is not to imply that pregneninolone, a synthetic substance not known to be produced in the tissues of any vertebrate, is the male sex hormones of fishes. An analogous compound, other than testosterone, however, may be the true hormone.

The question of the role of sex hormones in the gonad differentiation of the guppy is still left in confusion. Berkowitz (2) found that estrone would cause the develop-

ment of ovotestes in a high percentage of males. The expected result with androgenic treatment would then be the production of ovotestes in treated females. The eventual effects of testosterone and pregneninolone on the fish ovary were purely ones of inhibition, the latter compound having a more marked effect than the former. These results on the whole, were unlike the sex reversing effect of testosterone as reported for fish (*Xipophorus helleri*) (8), for amphibians (14, 15) and for the chick embryo (16).

In interpreting the action of pregneninolone on the testis it is important to consider the effect of prolonged treatment, because there is an initial precocious maturation of germ cells followed by a striking inhibition or exhaustion, apparently due to an inadequate replacement. The picture obtained depends upon duration of treatment. It is possible that the stimulating action is a direct one and the inhibition via the hypophysis.

The theory of Cutuly and Cutuly (17), concerning the role of gonadotropins and androgens in spermatogenesis of the rat, is in general, although not in all details, compatible with our results in Lebistes.

The partially androgenic properties of pregneninolone in mammals have been reported while this work was in press (18).

## SUMMARY

In Lebistes the effects of pregneninolone are purely androgenic and this compound fulfills the expected actions of an androgen better than testosterone propionate.

Pregneninolone fed to immature guppies from birth caused the precocious assumption of male secondary sex characters and completely prevented the development of any female secondary sex characters. Furthermore this compound induced a marked initial precocious maturation of existing germinal elements in sexually immature fish, which was followed after prolonged treatment by an exhaustion of testes to such an extent that only gonad remnants remained.

Testosterone propionate had effects like those of pregneninolone on secondary sex characters except for an estrogen-like inhibition of male red colors. Both testosterone and pregneninolone caused an inhibition, rather than a sex reversal of the ovary. Testosterone treated males contained testes which, like the mature testes treated with pregneninolone, were composed primarily of the later stages of spermatogenesis.

Progesterone, desoxycorticosterone acetate, adrenal cortical extract, and pregnandiol had no effects on sexual development in Lebistes.

The author wishes to express his gratitude for the helpful criticism of Dr. Robert Gaunt, at whose suggestion this and other similar work was undertaken. The author is also grateful to Dr. Harry A. Charipper for advice on histological interpretations.

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# INFLUENCE OF DELAYED RESORPTION ON SPECIFIC GONADOTROPIC AUGMENTATION

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NO ONE SERIOUSLY BELIEVES that the pituitary liberates its gonadotropic hormone once daily at a given time, yet the conventional assay of pituitary hormone is based on a single or at best double daily injection. The ovarian response so obtained is considerably less than that obtained by the process of prolonged delayed resorption or multiple divided dosage, yet this increased response obtained under more normal physiologic conditions is spoken of as the phenomenon of non-specific augmentation. Since it has been shown (1, 2) that the lessened response obtained by single daily injections is nothing more or less than the 'antagonism' phenomenon, which is elicited when pituitary extracts are rapidly resorbed, we consider the term non-specific augmentation unnecessary. There is, however, a type of augmentation produced by the combined action of pituitary extracts and prolan which has never been connected with the phenomenon of delayed resorption.

It is the purpose of this paper to study the influence of delayed resorption upon the augmentation produced by the combined action of pituitary extracts and prolan. Such an augmentation is also stated to exist for fractionated pituitary extracts, but we have been unable to produce it under conditions of assay assuring maximum response of each component. In order to study prolan augmentation, it was necessary to establish the influence of delayed resorption upon the response to prolan. The influence of delayed resorption upon the mare serum hormone was also studied.

## EXPERIMENTAL

The follicle stimulating hormone preparation[1] used in these experiments was part of a sample made in the laboratory of H. M. Evans and previously tested by us (2). The prolan was prepared in our laboratory and has been used in a number of experiments (3, 4). Twenty-two to 23-day-old litter mates were used in all experiments and the period of dosage was 4 days, the rats being killed on the 5th day.

*Delayed resorption of prolan (U. P.) and equine gonadotropin.* Like the pituitary gonadotropic hormone, prolan is precipitated by coppper under certain conditions. The copper is added at a slightly acid PH. By precipitating the copper as the hydroxide at PH 8.5, the prolan is removed from solution. In Experiments I and III, table 1, it is clearly demonstrated that prolan administered as the insoluble copper combination produces a greater ovarian response than is produced by the administration of the soluble prolan. This increase amounting to approximately 15 mg. is much less than is produced by administering the pituitary hormone under the same conditions. In Experiment III, table 1, the zinc combination of prolan produced an 8 mg. increase in

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[1] This preparation is currently known as the follicle stimulating hormone fraction. Since, however, in proper dosage it also produces luteinization, it would in our opinion be more properly called pituitary gonadotropic hormone.

ovarian weight. An increase of this magnitude is about the same as that produced by 5 divided doses per day (see Experiment I and II, table 1). These differences are probably significant as in each case the litter mate receiving divided dosage of the insoluble compound developed larger ovaries than its respective control. In Experiment IV, table 1, 1 mg. of prolan in solution was administered at a site removed from that at which 1 mg. of the insoluble copper combination was given. Evidence of the antagonism phenomenon is suggested.

While all these differences are slight when comparison with the pituitary hormone is made, a similar tendency is clearly indicated. It must be remembered, as the data in the table show, that prolan response is not proportional to dose level, and that the influence of copper is actually as great as is doubling or tripling the dose.

TABLE 1. INFLUENCE OF DIVIDED DOSAGE OR DELAYED RESORPTION UPON THE OVARIAN RESPONSE TO PROLAN OR EQUINE GONADOTROPIN

| Exp. No. | Total dose hormone per rat | No. of doses per day | Ovarian weight average |
|---|---|---|---|
| I | 0.3 mg. prolan | 1 | 30±1.9 |
| | 0.3 mg. prolan | 5[1] | 37±3.2 |
| | 0.3 mg. prolan+0.5 mg. Cu, pH 8.5 | 1 | 44±2.7 |
| II | 0.5 mg. prolan | 1 | 37±1.8 |
| | 0.5 mg. prolan | 5[2] | 48±3.0 |
| III | 0.5 mg. prolan | 1 | 37±1.8 |
| | 0.5 mg. prolan+1.0 mg. Zn, pH 8.5 | 1 | 45±3.3 |
| IV | 1.0 mg. prolan | 1 | 45±3.6 |
| | 1.0 mg. prolan+0.75 mg. Cu, pH 8.5 | 1 | 60±4.4 |
| | 1.0 mg. prolan (site removed)<br>1.0 mg. prolan+0.75 mg. Cu, pH 8.5 | 1 | 49±3.5 |
| V | 5 U equine gonad. | 1 | 27±1.8 |
| | 5 U equine gonad.+0.5 mg. Cu, pH 8.5 | 1 | 33±3.7 |
| | 10 U equine gonad. | 1 | 57±7 |
| | 10 U equine gonad.+0.5 mg. Cu (site removed) | 1 | 63±9 |
| | 10 U equine gonad.+0.5 mg. Cu, pH 8.5 | 1 | 69±5 |

[1] Doses at 9, 12 A.M.; 3, 6, 10 P.M.
[2] Doses at 9, 11 A.M.; 1, 3, 5 P.M.

Purified mare serum hormone was treated with copper in a manner strictly analogous to the precipitation of the urinary and pituitary gonadotropic extracts. This was given at two dosage levels. While the ovaries of the rats which received the copper combination are the larger the difference is not significant. Catchpole, Cole, and Pearson (5) have shown that in the rabbit the hormonal concentration of injected mare serum was reduced in half every 26 hours.

*Influence of delayed resorption on specific augmentation.* In order to minimize the influence of normal variation to response, 6 groups of 6 litter mates were used in Experiment VI, table 2, and 4 groups of 6 litter mates in Experiment VII, table 2. It will be noted that the dose of follicle stimulating hormone used, 0.02 mg., produced no significant increase in ovarian weight when administered by itself or when combined with copper. In another paper (2) we showed that 0.05 mg. of this preparation was required to produce a 50% increase in ovarian weight. At this dosage level copper produced a pronounced increase in response.

The results of Experiment VI and VII clearly show that 0.02 mg. follicle stimulating hormone increased the response to 0.3 mg. prolan by 33 to 35 mg., confirming pre-

vious work concerning this particular type of augmentation. When the pituitary preparation was administered as the copper combination, the increase was 53 mg. The further increase of 20 mg. is not significant on the basis of the standard deviation of the mean of each group. However, if a comparison of litter mates is made, five had larger, one had slightly smaller ovaries in the series which had received the copper combination. When the prolan alone was administered in copper combination, there was an added increase of 23 mg. in ovarian weight, which added increase is highly significant. Under these conditions the follicle stimulating hormone fraction produced a 22 mg. augmentation. This figure may be considered significant as each litter mate which received follicle stimulating hormone had larger ovaries than the corresponding

TABLE 2. INFLUENCE OF DELAYED RESORPTION OF EITHER COMPONENT UPON PROLAN—FSH SPECIFIC AUGMENTATION

| Material injected | Total dose hormone per rat | Mean ovarian weight | |
|---|---|---|---|
| | | Exp. VI | Exp. VII |
| | *mg.* | *mg.* | *mg.* |
| Control | — | 15±1.6 | |
| Prolan | 0.3 | 28±2.2 | 31±2.3 |
| Prolan+Cu | 0.3+0.5 Cu | | 54± 3.7 |
| FSH | 0.02 | 16±1.0 | |
| FSH+Cu | 0.02+0.5 Cu | 18±1.5 | |
| Prolan<br>FSH | 0.3<br>0.02 | 61±6 | 66±11 |
| Prolan+Cu<br>FSH | 0.3+0.5 Cu<br>0.02 | | 76± 9 |
| Prolan<br>FSH+Cu | 0.3<br>0.02+0.05 Cu | 81±9 | |

litter mates receiving only copper prolan. It may be concluded that the so-called specific augmentation is altered by the delayed resorption of either component and that it is relatively less if the prolan is more slowly resorbed and relatively more if the follicle stimulating fraction is more slowly resorbed.

## DISCUSSION

Since 0.02 mg. follicle stimulating hormone given in solution or in copper combination produces an insignificant (1 to 3 mg.) increase in ovarian weight, but when given with prolan produces a 30 to 50 mg. increase in ovarian weight, the phenomenon of specific augmentation is said to have occurred. In this connection it is interesting to note that if the amount of follicle stimulating hormone is raised to 0.05 mg. (given without prolan) (2), there is only a slight increase in ovarian weight (7 mg.) produced, but if this amount is given in connection with Cu, a 35 mg. increase in ovarian weight is produced. The added 0.03 mg., therefore, produces, if resorption is delayed, an effect equal to the effect produced by 0.02 mg. when added to prolan. The results may be stated in another way: an increment of follicle stimulating hormone superimposed on prolan will have the same order of influence it would have if superimposed on itself under conditions of delayed resorption. We are, therefore, forced to conclude that prolan, by some mechanism not understood, elicits a greater response from a given amount of follicle stimulating hormone, as does also delayed resorption. In addition our experiments show how this response with prolan is further influenced by delayed resorption of either component.

These experiments, as well as those previously reported, emphasize that the response to gonadotropic extracts is largely dependent upon the maintenance of a level

of hormone in the body and that the phenomena of specific and non-specific augmentation and antagonism are dependent wholly or in part upon the success or failure in maintaining the proper level or balance of levels.

SUMMARY

The so-called specific augmentation of prolan by follicle stimulating hormone is altered by the delayed resorption of either component and is relatively less if the prolan is more slowly resorbed and relatively more if the follicle stimulating hormone is more slowly resorbed.

An increment of follicle stimulating hormone superimposed upon prolan will have the same order of influence (augmentation) it would have if superimposed on itself under conditions of delayed resorption.

The so-called specific augmentation may be considered a response in which the 'antagonism' phenomenon, readily produced when pituitary extracts are rapidly resorbed, is not elicited.

The response to prolan as measured by ovarian weight increase is increased by delayed resorption but to a lesser degree than that manifested by the pituitary hormone. The mare serum hormone response is not appreciably affected.

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# THE MODE OF ADMINISTRATION AS AN INFLUENCE ON THE EFFECTIVENESS OF MARE SERUM IN HYPOPHYSECTOMIZED IMMATURE FEMALE RATS[1]

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THE OVARIAN RESPONSE to gonadotropic hormones may be markedly influenced by the frequency or route of injection. A single injection of human chorionic or anterior pituitary gonadotropin is not as effective as the administration of the same total dose in divided quantities. However, the gonadotropic material from pregnant mare serum has been shown to be just as effective on the immature rat ovary when administered as a single subcutaneous injection as when the injections are divided (1–4). Hamburger and Pedersen-Bjergaard (5) have shown that in experiments terminated at 100 hours a single subcutaneous or even a single intravenous injection, of a low dose of mare serum, was as effective as 5 subcutaneous injections spread over the first 48 hours.

Evans, Korpi, Pencharz and Simpson (6) obtained no difference in potency of mare serum hormone when administered either intraperitoneally or subcutaneously. Recently, Pencharz (7) has demonstrated in both normal and hypophysectomized immature female rats that the daily administration of a low dose of mare serum is more effective by the intraperitoneal than by the subcutaneous route. No difference in ovarian weight was obtained with a larger dose of extract. However, the ovarian histology was not reported.

The investigation here reported was undertaken to study the ovarian effects of injecting the same total dose of pregnant mare serum gonadotropin in 5 different ways to hypophysectomized immature female rats kept under identical experimental conditions.

## MATERIALS AND METHODS

Seventy-three immature female rats, ranging from 30 to 38 days in age, were hypophysectomized, at which time the vaginal orifice was closed in all cases. A 5-day postoperative interval was allowed to elapse before treatment was instituted. A total dose of 10 rat units (Cole-Saunders) of pregnant mare serum gonadin[2] was administered to all animals. This dosage was injected (*a*) subcutaneously, (*b*) intraperitoneally in equally divided doses once daily for 5 days, (*c*) intraperitoneally, (*d*) intravenously, (*e*) subcutaneously in a single injection. All animals were autopsied 120 hours after the initial injection unless otherwise stated.

The pituitary capsules were serially sectioned, stained with Masson trichrome and examined microscopically. Data from animals exhibiting pituitary fragments, no matter how small, were discarded.

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[2] Pregnant mare serum hormone, gonadin, was generously supplied by Dr. Donald Wonder, Cutter Laboratories.

At autopsy, the ovaries, adrenals and uteri were dissected out and weighed, the latter after the removal of intrauterine fluid. The ovaries were examined under the binocular dissecting microscope to ascertain grossly the response obtained. In addition, the ovaries of at least three-fourths of the animals in each series were microscopically examined after serial sectioning.

## RESULTS AND DISCUSSION

The subcutaneous administration of 2 R.U. of pregnant mare serum (PMS) once daily for 5 days (10 R.U. total dose) increased ovarian weight approximately four times. Ovaries of hypophysectomized controls after the same postoperative 10-day interval weighed 6 to 8 mg. This degree of stimulation compares with results obtained previously (8). When the injections were made intraperitoneally once daily for 5 days, there was a much greater average response (70.1 mg., table 1). Considerable individual variation resulted with the intraperitoneal injections and in 5 of the 17 cases the

TABLE 1. RESPONSES OF HYPOPHYSECTOMIZED IMMATURE FEMALE RATS TO PREGNANT MARE SERUM, GONADIN

| No. of rats | Body weight av. | | Mode of injection | Av. ovarian wt. | Range of ovarian wt. | Av. uterine wt. |
|---|---|---|---|---|---|---|
| | start | autopsy[1] | | | | |
| | gm. | gm. | | mg. | mg. | mg. |
| *Hypophysectomized control animals* | | | | | | |
| 6 | 77.1 | 67.2 | none | 6.6 | 5.4–8.0 | 20.3 |
| *Animals received 2 R.U. daily for 5 days* | | | | | | |
| 8 | 62.0 | 55.6 | subcut. | 27.1 | 13– 38 | 82.0 |
| 17 | 71.4 | 68.0 | intrap. | 70.1 | 23–183 | 120.2 |
| *Animals received a single injection of 10 R.U.* | | | | | | |
| 6 | 67.2 | 61.8 | intrav. | 48.5 | 37– 66 | 128.8 |
| 11 | 69.4 | 62.9 | subcut. | 43.7 | 21– 58 | 130.2 |
| 11 | 69.1 | 61.9 | intrap. | 55.5 | 38– 69 | 147.3 |

[1] All animals were autopsied 120 hours after the first injection or 10 days after hypophysectomy.

ovarian weight fell within the range of the weights obtained with subcutaneous injections. These results are in agreement with those of Pencharz (7) who, however, pointed out that this difference in effectiveness was not observable when a large dose was used. It has been suggested (7) that the failure of Evans et al. (6) to obtain a difference in potency by the two routes of administration may have been due to the large dose used. This increased effectiveness of the intraperitoneal route as compared to the subcutaneous route indicates a difference between PMS and the follicle stimulating hormone from the anterior pituitary (FSH). Fevold (9) reported FSH to be ineffective intraperitoneally and Evans, Simpson, Tolksdorf and Jensen (10) found FSH no more active when administered intraperitoneally than when injected subcutaneously.

A single injection of 10 R.U. of PMS administered either subcutaneously, intravenously or intraperitoneally was found to increase ovarian weight to approximately the same degree (table 1). It is to be noted that in all cases the single injection method increased ovarian weight to a greater extent than the same total dose administered subcutaneously in equally divided quantities once daily for 5 days.

Some of the differences in the effectiveness of PMS observed when the frequency or route of administration was varied has been attributed to slow absorption, to slow destruction in the body and to the fact that there is little if any excretion of this gonadotropic material (5, 11, 12).

A group of 8 hypophysectomized animals was killed at periods other than the routine 120-hour period. A single intraperitoneal injection of 10 R.U. was given. One ovary was removed at 72 hours after injection and the animals were killed 24 hours later. The ovarian weights averaged 25.9 mg. at the 72-hour period and 35.6 mg. 96 hours after the injection. The maximum ovarian weight appeared to have been reached at the 96-hour interval since the average ovarian weight for a single ovary at 120 hours was 27.7 mg. Ovarian weight was also determined in hypophysectomized animals autopsied 240 hours after a single subcutaneous or intraperitoneal injection of 10 R.U. of PMS. The weight of the ovaries averaged 12.9 mg. (4 rats) following subcutaneous administration and 8.0 mg. (6 rats) after intraperitoneal injection. Cartland and Nelson (13) observed that maximum ovarian response in normal rats occurred 96 hours after the initial injection. These investigators used 3 equal subcutaneous injections at daily intervals.

The increase in uterine weight corresponded to the degree and type of ovarian stimulation obtained. Maximum uterine stimulation was not attained following daily subcutaneous injections but was observed after other modes of injection.

Administration of PMS did not prevent the typical adrenal weight loss resulting after hypophysectomy.

It was deemed of interest to study the ovarian histology to determine whether the mode of injection altered the nature of the response. The subcutaneous injection of 2 R.U. of hormone administered once daily for 5 days produced a marked luteinization of the thecal tissue. Small follicles with or without antra were also present in the ovaries and in a few cases normal vesicular follicles of medium size were observed.

The daily intraperitoneal injection method increased ovarian weight in some animals only to the same degree as that observed following the daily subcutaneous injections. In these cases, the intraperitoneal route of administration did not alter the ovarian histology. The marked thecal luteinization and the presence of small and a few medium sized vesicular follicles was again observed. However, the daily intraperitoneal injections did increase ovarian weight in other animals to a range well above that resulting from subcutaneous injections and a corresponding difference in histology was observed. These larger ovaries contained normal vesicular follicles of medium and large size. Thecal luteinization was less pronounced. In addition, granulosa luteinization was observed in 3 cases and corpora lutea were present in the ovaries of two other animals. Previously (8) it was observed that granulosa luteinization occurred in the ovaries of hypophysectomized immature rats following subcutaneous injections but that a dose of at least 8 R.U. daily for 5 days was necessary to produce the response. The ovarian weights averaged 145 mg.

The administration of 10 R.U. of PMS as a single injection caused thecal luteinization as well as the stimulation of normal vesicular follicles to medium size and a few to larger size regardless of the route of injection. Corpora lutea were observed following the single intraperitoneal injection in 4 of 11 rats and following the intravenous injection in 3 of 6 rats. Corpora lutea did not result from subcutaneous hormone administration of 10 R.U. in a single injection or in divided daily injections. This formation of corpora lutea appeared to be the result of the mode of injection rather than the degree of stimulation since ovarian weight increase was comparable regardless of the route of the single injection.

Ovaries of 8 animals were examined microscopically for corpora lutea at 72 and 96-hour intervals after a single intraperitoneal injection of 10 R.U. Corpora lutea were observed in the ovary of one rat at the 72-hour period. Other ovaries examined after 72 hours exhibited medium sized vesicular follicles and some thecal luteinization. At the 96-hour interval, the ovaries of 2 animals had corpora whereas in other cases a large proportion of the ovary was composed of good sized vesicular follicles. Thecal luteinization was slight.

## SUMMARY

The ovarian response elicited in hypophysectomized immature female rats by administering the same total dosage of equine gonadotropin in 5 different ways was studied. A single injection, regardless of the route of administration, exhibited more potency than divided subcutaneous injections. However, the greatest average ovarian response was produced by the daily intraperitoneal injection method.

Corpora lutea formation resulted in some animals following intraperitoneal or intravenous injections but was not observed after subcutaneous hormone administration.

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# EFFECT OF TESTOSTERONE PROPIONATE, ESTRADIOL BENZOATE AND DESOXYCORTICOSTERONE ACETATE ON THE KIDNEYS OF ADULT RATS[1]

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DURING THE COURSE of our experiments on the effect of testosterone on the vascular system, it was noted that testosterone propionate caused an increase in the size of the kidneys of normal rats (1). Korenchevsky, Dennison and Kohn-Speyer (2, 3, 4) had reported a decrease in the weight of the kidneys of male rats following castration and an increase in kidney weights following injections of androgenic urine extracts. More recently, Selye (5) had reported kidney enlargement in normal female mice after the injection of large doses of testosterone propionate.

While our experiments were in progress, Selye (6) reported increases in the kidney weight of normal male and female rats injected with testosterone propionate, estradiol, desoxycorticosterone acetate and progesterone, and Korenchevsky and Ross (7) described hypertrophy of the kidneys of normal and ovariectomized female and of castrated male rats following the administration of testosterone esters and androsterone; they did not consider the enlargement in their small series of normal male rats to be definitely "supernormal."

The present investigation was designed in an effort to study the effect of testosterone propionate, alone and in combination with estradiol benzoate and desoxycorticosterone acetate, on the kidneys of normal adult male and female rats. The influence of dosage and the mechanism of the production of the renal enlargement have been given special attention.

## METHODS

One hundred and two adult male and female albino rats of the Wistar strain weighing from 120 to 250 gm., were used in these experiments. Liberal amounts of Purina Dog Chow and water were constantly available. Litter mates were apportioned to the various groups in as nearly equal proportions as possible.

Testosterone propionate, estradiol benzoate and desoxycorticosterone acetate[2] were administered subcutaneously in dilutions so adjusted that the total daily dose was dissolved in 0.4 cc. of sesame oil. When two substances were given to the same animal they were injected at different subcutaneous sites.

Eighteen male and ten female rats served as controls; they received 0.4 cc. of

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[1] The hormones used in this study were supplied through the courtesy of the Ciba Pharmaceutical Products, Inc., Summit, N. J.

[2] Testosterone propionate, perandren; estradiol benzoate, ben-ovocylin; desoxycorticosterone acetate, percorten.

sesame oil daily for 14 to 42 days. Twenty-one male and eight female animals received testosterone propionate (0.5 to 12.5 mg. daily) for 14 to 42 days; 10 males and 3 females received estradiol benzoate (0.1 to 0.4 mg. daily) for 21 to 42 days; 18 males and 4 females were given testosterone propionate and estradiol benzoate, in varying doses, for 21 to 42 days; 5 males were given desoxycorticosterone acetate (1.0 mg. daily) for 42 days; and 5 males received testosterone propionate (0.5 mg.) and desoxycorticosterone acetate (0.5 mg.) for 42 days.

All animals were killed 24 hours after the last injection. Both kidneys were weighed immediately in the fresh state. One kidney was macerated and placed in an oven for the subsequent determination of its dry weight. In the 21-day experiments,

TABLE 1. KIDNEY WEIGHTS OF ADULT MALE RATS AFTER THE INJECTION OF STEROID HORMONES[1]

| Group | Hormone | Daily dose | No. days | No. rats | Mean body Wt. | Mean kidney weights | | | | Mean water content of kidney | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Fresh (2 kidneys) | | Dry (1 kidney) | | | |
| | | mg. | | | gm. | gm./kg. | | gm./kg. | | % water | |
| 1 | Sesame O. (Control) | 0.4 cc. | 14–42 | 18 | 215 | 6.92±0.07 | 0.00[2] | 0.815±0.02 | 0.00[2] | 76.06±0.18 | 0.00[2] |
| 2 | T. P.[3] | 0.50 | 21 | 3 | 192 | 8.02±0.20 | 3.50 | 0.940±0.02 | 3.12 | 76.60±0.14 | 1.67 |
| 3 | T. P. | 1.00 | 21 | 4 | 174 | 9.67±0.28 | 6.48 | 1.061±0.02 | 6.72 | 77.45±0.15 | 4.06 |
| 4 | T. P. | 1.00 | 42 | 6 | 228 | 9.17±0.13 | 10.43 | 1.054±0.01 | 9.27 | 77.36±0.18 | 3.50 |
| 5 | T. P. | 10.00 | 21 | 4 | 265 | 8.89±0.14 | 8.54 | — | — | — | — |
| 6 | T. P. | 12.50 | 14 | 4 | 181 | 8.38±0.39 | 2.45 | — | — | — | — |
| 7 | E. B. | 0.01 | 21 | 3 | 146 | 7.70±0.10 | 4.35 | 0.820±0.01 | 0.23 | 78.51±0.15 | 6.98 |
| 8 | E. B. | 0.04 | 21 | 4 | 122 | 8.50±0.16 | 6.44 | 0.894±0.02 | 2.59 | 79.04±0.14 | 8.78 |
| 9 | E. B. | 0.04 | 42 | 3 | 191 | 8.43±0.17 | 5.64 | 0.875±0.02 | 1.79 | 79.39±0.22 | 8.64 |
| 10 | T. P.<br>E. B. | 0.50<br>0.04 | 21 | 4 | 154 | 9.61±0.25 | 7.09 | 0.992±0.04 | 3.07 | 79.03±0.08 | 10.17 |
| 11 | T. P.<br>E. B. | 1.00<br>0.01 | 21 | 4 | 160 | 10.38±0.13 | 16.41 | 1.073±0.02 | 8.87 | 79.50±0.14 | 10.16 |
| 12 | T. P.<br>E. B. | 1.00<br>0.04 | 21 | 7 | 146 | 10.44±0.18 | 12.31 | 1.044±0.02 | 7.53 | 79.68±0.12 | 11.05 |
| 13 | T. P.<br>E. B. | 1.00<br>0.04 | 42 | 3 | 204 | 11.99±0.30 | 11.29 | 1.228±0.02 | 13.32 | 79.80±0.40 | 9.74 |
| 14 | D. A. | 1.00 | 42 | 5 | 250 | 8.43±0.21 | 4.64 | 0.966±0.02 | 4.06 | 77.11±0.29 | 2.11 |
| 15 | T. P.<br>D. A. | 0.50<br>0.50 | 42 | 5 | 232 | 8.89±0.18 | 7.00 | 1.029±0.01 | 8.29 | 76.60±0.21 | 1.18 |

[1] Statistical analysis by method of Hartung & Bruger (8), except that (N−1) was used instead of N.
[2] T. P.—Testosterone Propionate
E. B.—Estradiol Benzoate
D. A.—Desoxycorticosterone Acetate
[3] Difference between the means of the control and experimental groups divided by the standard error of the difference of the means. Results are considered to vary significantly from the normal when this ratio is greater than three.

the other kidney was fixed in formalinized Zenker's solution for histological study. Hematoxylin and eosin stains were used for the histological preparations.

These studies were carried on in a series of 4 separate experiments, each with adequate controls, and the results have been grouped together for the sake of clarity.

## RESULTS

A comprehensive analysis of the kidney weights, including a statistical study, is presented in tables 1 and 2.

*Effect of testosterone propionate.* The kidneys of the male rats injected with testosterone[3] (*groups 2, 3, 4, 5*, table 1) showed a statistically significant increase in fresh weights when compared with the control group (*group 1*, table 1). In *group 6* the increase was considered suggestive but not definite. The most significant increases occurred in *groups 3, 4, 5* after doses of 1 to 10 mg. daily. Smaller doses (*group 2*), and larger doses (*group 6*) gave less significant results. The most efficient dose of those employed appeared to be 1 mg. daily. The increase in dry weights was of the same order of significance as for the wet weights in the 3 groups studied. There was a moderate increase in the renal water content.

[3] Hereafter in this paper testosterone will be used to indicate testosterone propionate.

The kidneys of the females injected with testosterone (*groups 2, 3*, table 2) showed an effect similar in character to that observed in the males, although the increase in weights, when compared with the controls (*group 1*, table 2) was smaller in degree. Of the doses employed, 10 mg. daily gave a definitely significant increase while the increase following 1 mg. was not considered to indicate more than a trend.

*Effect of estradiol benzoate.* Although the fresh kidney weights of the male rats injected with estradiol benzoate (*groups 7, 8, 9*, table 1) showed an increase over the controls, this increase was less than that obtained after optimal doses of testosterone given over the same period of time. The large increase in water content in these groups, however, was sufficient to account for most of the increase in the wet weights. Analysis of the dry weights revealed relatively insignificant increases over the control

TABLE 2. KIDNEY WEIGHTS OF ADULT FEMALE RATS AFTER THE INJECTION OF STEROID HORMONES

| Group | Hormone | Daily dose | No. days | No. rats | Mean body wt. | Mean kidney weights | | | | Mean water content of kidney | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Fresh (2 kidneys) | | Dry (1 kidney) | | | |
| | | *mg.* | | | *gm.* | *gm./kg.* | | *gm./kg.* | | *% water* | |
| 1 | Sesame O. (Controls) | 0.4 cc. | 21–42 | 10 | 174 | 7.50±0.15 | 0.00[1] | 0.831±0.013 | 0.00[1] | 77.68±0.10 | 0.00[1] |
| 2 | T. P. | 1.00 | 21 | 3 | 188 | 8.70±0.66 | 1.23 | 1.953±0.039 | 2.00 | 77.68±0.67 | 1.23 |
| 3 | T. P. | 10.00 | 21 | 5 | 193 | 9.33±0.18 | 4.86 | — | — | — | — |
| 4 | E. B. | 0.04 | 21 | 3 | 161 | 8.21±0.11 | 2.95 | 0.855±0.019 | 0.70 | 79.23±0.26 | 3.71 |
| 5 | T. P.<br>E. B. | 1.00<br>0.04 | 21 | 4 | 165 | 9.97±0.55 | 2.95 | 1.050±0.061 | 2.95 | 79.08±0.22 | 3.90 |

[1] Difference between the means of the control and experimental groups divided by the standard error of the difference between the means. Results are considered to vary significantly from the normal when this ratio is greater than three.

values. Similar, but less striking, results were found in the female group (*group 4*, table 2) receiving estradiol benzoate.

*Effect of desoxycorticosterone acetate.* The fresh kidney weights of the male rats given desoxycorticosterone acetate (*group 14*, table 1) were increased significantly over the controls but to a less extent than for those receiving testosterone in equal dosage (*group 4*, table 1). The increase in dry weight was of the same order of significance as for the fresh weights, since there was a relatively small increase in water content.

*Effect of testosterone plus desoxycorticosterone acetate.* The kidney weights following simultaneous administration of testosterone and desoxycorticosterone acetate in 0.5 mg. daily doses were definitely increased (*group 15*, table 1), but to a lesser extent than the increase produced by 1 mg. of testosterone given alone (*group 4*, table 1). The increase may be explained by a simple additive effect of the two substances in the doses used. The dry weights were increased to a degree similar to the wet weights and there was an insignificant increase in water content.

*Effect of testosterone plus estradiol benzoate.* The greatest increases in kidney weights occurred after the simultaneous administration of testosterone and estradiol benzoate in the male rats. The most significant increase was noted in the groups given 1 mg. testosterone and 0.01 mg. estradiol benzoate daily for 21 days (*group 11*, table 1), although only slightly less significant values resulted when the dose of estradiol benzoate was raised to 0.04 mg. (*group 12*, table 1). A less marked, but significant increase occurred after 0.5 mg. of testosterone and 0.04 mg. of estradiol benzoate (*group 10*, table 1).

The wet weights, dry weights and water content of these groups represent a roughly additive effect of the increases obtained when the hormones were injected alone in the same doses.

The results for the female group (*group* 5, table 2) are similar in character to those observed in the male groups, but less marked in degree. Although the mean values appear to represent large increases in the female groups, the significance of the increase was diminished by the relatively large variation within the groups.

*Histological findings.*[4] The histological sections of the kidney from the groups receiving the hormones for 21 days revealed no marked changes in structure when compared with the control group.

The cells of the convoluted tubules appeared to be hypertrophied in the kidneys of animals receiving testosterone, although the parietal lamina of Bowman's capsule showed no hypertrophic changes. Some of the glomerular endothelial cells showed slight thickening. There was no increase in the connective tissue stroma.

The sections from rats receiving estradiol benzoate revealed occasional spaces filled with pink staining homogeneous material in various portions of the cortex. These areas appeared to be in the peritubular spaces and were interpreted as collections of edematous fluid. They were noted more frequently in the male and female groups receiving 0.04 mg. estradiol benzoate daily (*group* 8, table 1; and *group* 4, table 2). The convoluted tubules showed no appreciable changes in these groups.

There was a relative hyperemia of the glomeruli and of the medullary and cortical vessels of some of the kidneys from animals receiving testosterone and estradiol benzoate simultaneously. No other definite structural changes were observed in these groups, although a diffuse renal enlargement was evident on gross examination.

The histological changes noted above appeared to a similar degree in the kidney section from both sexes.

## DISCUSSION

It is apparent from the results presented that testosterone, estradiol benzoate and desoxycorticosterone acetate, when administered in efficient dosages, produce a definite increase in the weight of the kidneys of adult male and female rats. These findings are in agreement with those of Selye (6). Although Korenchevsky and Ross (7) did not consider the enlargement in their small group of testosterone treated male rats to be significant, we have demonstrated significant increases in both sexes as did Selye. In fact, our results indicate a consistently greater effect in males than in females.

The observation of an optimal effective dosage of testosterone is consistent with the findings of Selye in mice (5). He found a daily dose of 0.5 to 1.0 mg. to be more effective than 5 mg. It seems probable that for estradiol benzoate, too, there is an optimal effective dose for the production of renal changes in rats. Although only two doses were employed in our estradiol studies, it is evident that the 0.01 mg. dose was nearly as effective as the 0.04 mg. dose. When given simultaneously with testosterone, 0.01 was slightly more effective than 0.04 mg.

The apparent synergistic effect of the simultaneous administration of testosterone and estradiol benzoate, as well as desoxycorticosterone acetate, may be explained by the simple addition of the individual effects of these steroids. When the optimal dose of testosterone is reached, however, additional testosterone produces no further renal enlargement although the simultaneous administration of one of the other steroids produces additional enlargement. These observations emphasize the importance of the hormonal balance in the physiologic economy of the body.

It is of interest that the increase in wet weight produced by testosterone and desoxycorticosterone acetate could be accounted for to only a small extent by the increase in water content, whereas the changes produced by estradiol benzoate could be

[4] We wish to thank Dr. Howard G. Benjamin of the New York Post-Graduate Hospital for his assistance in reviewing the histological sections.

explained primarily on that basis. This would indicate that testosterone and desoxycorticosterone acetate produce an increase in renal substance and that estradiol benzoate simply produces retention of fluid in the renal tissues. The hypertrophy of the convoluted tubules noted in the kidneys of testosterone treated rats and not in those given estradiol benzoate and the collections of edema fluid noted in the kidneys of estradiol benzoate treated animals but not in those given testosterone lend further support to these observations.

The histological findings are apparently similar in character to those reported by Korenchevsky and Ross (7) although the changes produced by estradiol benzoate are relatively minor when compared to their finding following the prolonged administration of estradiol benzoate-butyrate and estradiol dipropionate. The possibility that the water retention produced by estradiol esters represents a 'toxic' effect (7), must be given consideration although further studies appear to be necessary. It is our impression from these studies that the renal enlargement produced by testosterone is due primarily to hypertrophy and perhaps some hyperplasia of the convoluted tubules.

The increase in parenchymatous renal tissue produced by testosterone and desoxycorticosterone acetate infers the possibility of favorably influencing renal function through the administration of these steroids. The importance of dosage, ratio and side effects must be carefully considered in the clinical trial of these substances for effects on renal function.

## SUMMARY

Significant increases in the kidney weights of adult male rats have been produced by the administration of testosterone propionate, estradiol benzoate and desoxycorticosterone acetate. Similar but less significant changes have been produced by testosterone propionate and estradiol benzoate in adult female rats. Of the steroids studied, testosterone propionate appears to have the most marked effect.

Evidence has been presented to indicate that testosterone propionate and desoxycorticosterone acetate produce an increase in renal substance and that estradiol benzoate simply produces retention of fluid in the renal tissues.

An optimal effective daily dose of testosterone propionate has been demonstrated.

The simultaneous administration of testosterone propionate and estradiol benzoate, as well as desoxycorticosterone acetate, has been shown to produce further renal enlargement which represents a roughly additive effect of the individual substances.

The possible clinical significance of these observations has been discussed.

We wish to acknowledge the technical assistance of Mr. Julius Wilensky in these studies.

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# EFFECT OF REMOVAL OF OVARIES AND ADRENALS ON OPENING OF THE VAGINA IN THE ALBINO RAT

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FROM THE WELL-KNOWN ROLE of the estrogenic hormone in causing the vagina to open in certain experimental animals, it may be inferred that the same factor is involved in normal animals. Since this occurs in ovariectomized rats at approximately the same stage of development as the normal, it becomes necessary to seek a source of estrogenic hormone outside of the ovary or to look for some other cause.

Several investigators (1, 2, 3) have found that adrenalectomy disturbs or suspends the estrous cycle in the rat. In cases of adreno-genital syndrome Simpson, de Fremery and Macbeth (4) reported an excessive excretion of the male hormone. Estrogenic substances were found in the blood and urine by Frank, Goldberger and Salmon (5) following surgical removal of the ovaries, physiological menopause, and x-ray castration. Parkes (6) has suggested that the adrenals may be the source of these hormones. In support of this Engelhart (7), and Callow and Parkes (8) have observed that extracts of the adrenal cortex contain estrogenic material. The actual isolation of estrone from the adrenal gland has been reported (9). It is also of interest that according to Burrill and Greene the adrenals are probably responsible for the maintenance of the prostate of the immature male castrate rat in a normal or nearly normal condition (10), and also for the transitory stimulation of the female prostate (11). From a different line of investigation it was observed by Davidson and Moon (12) that androgenic activity of the adrenals followed the administration of adrenocorticotropic hormone to young castrated rats. In the immature female the opening of the vagina followed similar treatment (13).

These reports, along with a number of others, suggested that it would be of interest to investigate the factors involved in the establishment of the introitus vaginae in the adrenalectomized rat.

## PROCEDURE

Observations were carried out on 4 groups of animals: *a*) unoperated controls, *b*) castrates, *c*) adrenalectomized animals and *d*) adrenalectomized castrates. For the experimental groups females between the ages of 27 and 40 days were etherized and bilateral removal performed by the lumbar approach. An electric cauterizing tool was employed, care being taken to remove the surrounding connective tissue to minimize regeneration. All animals were placed in a room of relatively constant temperature. Controls and castrates were maintained on a diet of Purina Dog Chow and tap water. Chow and Rubin and Krick (14) salt solution were first provided for the adrenalectomized groups. As observed previously by Weiser and Norris (15) the survival time of young animals given the Rubin and Krick solution was short. Since reports (16, 17) indicated that the early death of these animals might be due to the

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toxicity of potassium, although according to Ingle, Nilson, and Kendall (18) the concentration of the latter does not determine the state of adrenal insufficiency, it was thought advisable to eliminate this factor as far as practicable. A potassium-low modification of the Harrison and Darrow (19) diet was fed in place of the Purina Chow and sodium citrate was substituted for the potassium chloride in the Rubin and Krick solution. Sodium citrate was employed since Allers and Kendall (20) obtained better results when this was added to the diet than when sodium chloride alone was used. Inasmuch as the rats seemed to crave meat, as evidenced by the survivors eating those dying during the experiment, liver was fed twice weekly. The diet and salt mixtures are given in table 1.

In view of reports (21, 22) that adrenalectomized as well as normal rats are able to select food conducive to favorable growth and reproduction, 13 animals were given

TABLE 1

Low Potassium Modification of the Harrison and Darrow Diet

| | parts |
|---|---|
| Casein | 20 |
| Sucrose | 25 |
| Dextrose | 37 |
| Lard | 22 |
| Cod liver oil | 3 |
| Yeast | 5 |
| F.R.L. salt mixture (Modified) | 4 |
| $CaCO_3$ | 1 |

Low Potassium Modification of the Rubin and Krick Salt Solution

| | parts |
|---|---|
| NaCl | 7.0 |
| $CaCl_2$ | 0.33 |
| Na Citrate | 1.0 |
| MgCl | 0.15 |
| Tap water | 1,000.0 |

Low Potassium Modification of the F.R.L. Salt Mixture (30)

| | parts |
|---|---|
| Ca Citrate | 30.6 |
| $Ca(H_2PO_4)_2$ | 11.3 |
| $Na\ HPO_4$ | 21.9 |
| $Na_2Citrate$ | 12.5 |
| $NaCl_3$ | 7.7 |
| $CaCl_2$ | 6.9 |
| $MgCl_4$ | 3.3 |
| Fe Citrate | 1.3 |
| NaF | 0.5 |
| $CaSO_4$ | 0.1 |
| $NaHCO_3$ | 10.0 |
| $MnSO_4$ | 0.15 |
| NaI | 0.04 |
| $Na_2Al_2(SO_4)_4$ | 0.09 |

unrestricted access to containers of potassium-low diet, Purina Chow, yeast, liver, dry salt, salt solution, and water. In addition to this, bread, which Swann (23) had found beneficial, was offered to a group of 5 at the starting period and later added to the diet of a number of animals. Eight animals were given injections of riboflavin and vitamin B; another group of 6 received glucose.

The animals were examined daily during the period in which the establishment of vaginal introitus could be expected, as determined by preliminary observations. Some groups were observed daily during the entire experiment. Otherwise they were examined at 5-day intervals when the body weights were recorded. Some animals were weighed daily for variable periods. When the vagina was observed to be open the age, weight and body length, taken from the tip of the nose to the anus, were recorded. The body length is important since Engle, Crafts and Zeithaml (24) have found it to be less variable at the time of rupture of the vaginal membrane than either age or weight. Varying degrees of opening were observed and recordings of three types were made: 1) approximately normal, 2) a definite orifice but about one-half normal size and 3) only the slightest indication of an opening.

The tissues to be examined microscopically were fixed in Bouin's fluid and stained with hematoxylin and eosin.

## RESULTS

In the 24 unoperated animals at the time of the establishment of the introitus vaginae the average age was 50.0 days, the average weight 100.0 gm., and the average

body length 16.1 cm. The results above for age correspond fairly well with the report of Curtis and Doisy (25) in which the average, calculated from their data, was about 44.6 days.

Four of the 22 castrates showed *type 2* opening, 16 *type 1*, but 2 did not exhibit orifices until they were well over 90 days of age and 200 gm. in weight. The averages, for age, weight, and length for this group, exclusive of the two just mentioned, were: 56.1 days, 127.6 gm., and 17.4 cm. It is thus seen that removal of the ovaries retards somewhat vaginal introitus and also it is usually smaller than that seen in normal animals.

Of 24 adrenalectomized animals, the 17 which survived showed vaginal opening

TABLE 2

| Glands removed | Type Opening | Age operated | No. of animals | Averages at time of Age | Weight | Length |
|---|---|---|---|---|---|---|
| | | *days* | | *days* | *gm.* | *cm.* |
| None | 1 | — | 24 | 50.0 | 100.3 | 16.1 |
| Ovaries | 1 and 2 | 27–40 | 22 | 56.1 | 127.6 | 17.4 |
| Adrenals | 1 | 27–40 | 17 | 51.7 | 90.1 | 15.5 |
| Ovaries and adrenals | no opening | 27–40 | 13 | 118.2[1] | 123.3[1] | 18.2[1] |
| | 3 | under 35 | 14 | 96.0 | 153.5 | — |
| | | over 35 | 15 | 79.3 | 140.0 | — |
| | 2 | under 35 | 6 | 116.3 | 145.5 | 19.5 |
| | | over 35 | 14 | 87.6 | 132.7 | 18.6 |
| | 1 | under 35 | 15 | 98.7 | 137.4 | 18.8 |
| | | over 35 | 14 | 63.2 | 119.4 | 17.6 |
| | 1, 2 and 3 | under 35 | 35 | 100.5 | 146.0 | 19.1 |
| | | over 35 | 43 | 76.8 | 130.9 | 18.1 |
| | 1, 2 and 3 | 27–40 | 78 | 87.6 | 137.8 | 18.5 |

[1] Recordings made after death.

at approximately the same age as the normal. The averages for these are 51.7 days, 90.1 gm. and 15.1 cm.

One hundred and fifty-seven females were adrenalectomized and ovariectomized. Ninety-three survived to show vaginal introitus or to reach an age of 75 or more days. Of these 93 animals, 13 died without exhibiting openings, in 2 the membrane was still patent at ages of 100 and 130 days when observations were discontinued, while the remaining 78 showed openings of one of three types. In many cases in which the first indication of introitus was regarded as *type 3*, some developed *type 2* or *type 1* within a few days. In 30 animals, however, no further development occurred for several weeks; since the opening was questionable no record of body length was made. The animals were divided arbitrarily into 2 age groups: those operated at ages under 35 days, and those operated at ages over 35 days. These results are shown in table 2.

It is apparent that the 13 rats which died before the vaginal membrane had ruptured were lighter in weight and only slightly longer than the castrates although they were much older. It is possible that if they had survived a short time longer they would have shown openings. All others of the adrenalectomized castrate group were 75.3% older, 37.4% heavier, and 14.8% longer than normal animals at the time of vaginal introitus. Compared with castrates they were 56.3% older, 8.0% heavier, and 5.2% longer. Since the length of 30 animals was not taken, these figures do not represent accurately the whole group, but they do indicate that in general these animals were larger and much older at opening time than normal or castrate rats.

To each of 8 rats which had not opened at ages of 110 days an adrenal implant was given, followed by a second 10 days later. Five of these animals showed introitus following the second implant while the other 3 died without further development.

While the potassium-low diet and potassium-free salt solution were an improvement over Purina Chow and Rubin and Krick salt solution, growth and survival were still subnormal. Approximately 60% of the adrenalectomized animals survived 50 days or more while about 21% died within 20 days. That age is a factor is shown by the fact that of those adrenalectomized below 35 days, 50% survived 50 days or more and 28% died within 20 days. Of those operated above 35 days of age, 69% survived 50 days or more and 17% died within 20 days.

As a further check on the special diet and the degree of adrenal insufficiency, 42 animals were replaced on the stock diet with the result that a number lost weight and died within a few days. When the survivors were again given the special diet they regained their lost weight. Twenty-eight continued to survive on the stock diet but few of these grew well or looked normal. Further attempts to improve the nutritional status of the animals by allowing them to select their own food or by the injection of riboflavin, vitamin B, and glucose failed to result in any appreciable improvement.

At autopsy small masses found near the site of one or both adrenals in 20 long term survivors proved to contain cortical or cortical-like cells when examined microscopically. In spite of the presence of this tissue the time of vaginal introitus was delayed, occurring at ages ranging from 50 to 158 days with an average of 83 days.

## DISCUSSION

The results presented here showing that the removal of the ovaries and adrenals, while having a variable effect, delays the opening of the vagina, lends support to the previously mentioned evidence that the adrenals may produce estrogenic hormones. These substances are probably responsible for the establishment of vaginal introitus in the ovariectomized rat. Since the vaginal membrane ruptured eventually in the majority of the animals even after the loss of both the ovaries and adrenals this may indicate the presence of accessory or regenerated adrenal tissue, or still other cells capable of producing estrogenic material. However, since the age at which adrenalectomy is performed is a factor there remains the possibility that other developmental agents are involved.

In a similar manner the superior growth and survival of some animals on the special diet and the continued survival when replaced on the stock diet may be attributed to regenerated or accessory tissue. Great care was exercised in the removal of the adrenals and in the few cases where the capsule was ruptured, the entire site was carefully cauterized to ensure as far as possible the complete removal or destruction of the gland. In spite of this, nests of cortical or cortical-like cells were found in 20 long term survivors.

The nature of the tissue in question is uncertain. In some cases it may be composed of juvenile cortex described by Howard (26), while in other cases it may be composed of different types of cortical cells. Which type or types of cortical cells produce the estrogenic material remains to be demonstrated. The relative amounts of the different cortical cells may explain why some animals which had a better rate of growth and a more healthy appearance than the average for the experimental group manifested an even greater retardation in vaginal opening than did others showing obvious signs of cortical insufficiency.

The subnormal growth and survival of the experimental animals is of considerable importance. While the special diet and salt solution improved the general condition

of the animals it is apparent that normal metabolic functions were greatly disturbed. Conditions in young animals therefore seem to be different than those found in older rats since the latter, when given adequate salts, appear normal and the absorption of carbohydrates and fats from the intestine and the formation of liver and muscle glycogen fall within normal limits (27, 28, 29). Whether this represents an essential change in metabolic functions conditioned by age is not clear. The liver in these animals was small at autopsy which might indicate an alteration in absorption or utilization of food substances. Just how much these metabolic functions affect the reproductive system is uncertain but the accomplishment of normal growth in young adrenalectomized rats without cortical extract would undoubtedly help clarify the adrenogenital relationship.

## SUMMARY

Removal of the adrenals and the ovaries retards and in some cases inhibits vaginal opening in rats. This lends further support to the view that the adrenals produce estrogenic substances. Normal growth was not obtained and the discovery of a diet capable of promoting normal growth and survival in young adrenalectomized rats will quite probably help clarify the adreno-genital relationship.

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# THE EFFECT OF STILBESTROL IN EARLY PREGNANCY[1]

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IN 1938 PARKES, DODDS, AND NOBLE (1) reported that 4–4 dihydroxy-$\alpha$: diethyl-stilbene, stilbestrol, was orally effective in the prevention of implantation in both rats and rabbits. Inasmuch as their report did not include the effects of stilbestrol upon the ova, and earlier investigations (2, 3) had shown the capacity of other estrogens to tube-lock or accelerate the rate of transport, this study was undertaken to determine the history of the ova following stilbestrol injections.

## PROCEDURE

Stilbestrol, dissolved in sesame oil, was injected subcutaneously into mated albino mice 8 to 12 hours after finding the vaginal plug (a.v.p.). Injections were daily with certain exceptions noted in table 1. Three groups of animals were injected with 1.0 mg., 0.5 mg. and 0.005 mg. respectively.

When autopsied, the reproductive tracts were examined for vascularity and spontaneous activity and the number, color and size of the corpora lutea noted. The uteri and oviducts were checked for ova or early embryos, and, in a few cases, the vaginal contents were examined.

## RESULTS

*Effects of daily injections of 1 mg. stilbestrol.* In this group stilbestrol not only accelerates the rate of tubal passage of the ova but also has a definite toxic effect on the ova. All ova, whether collected from the tubes or from the uteri, were either delayed in development, fragmenting, or showed definite signs of degeneration, such as irregularity in shape or a peculiar yellowish color. The eggs from mice 5 and 8 were still in the tubes at 32 and 36 hours a.v.p., but this is probably too soon to expect acceleration, as only 21 and 28 hours had elapsed since the injections. However, after 50 hours a.v.p. there is good evidence of acceleration. When *mouse* 6, which had received 2 injections, was autopsied 53 hours a.v.p., an empty zona was found in the lower uterus. Then the vagina was flushed to determine whether or not ova can escape from the uterus, and one degenerate ovum was obtained. This is the only record we have in this laboratory of an ovum being discovered in the vagina, and this probably would occur only in acceleration experiments.

Two injections do not seem necessary for acceleration; *mouse* 4 had had only one injection, yet the eggs were in the uterus 60 hours a.v.p. It may be argued that this is about normal, for mating doubtless took place several hours earlier. However, all ova recovered were 1-celled ova. Normally, by this time, the ova have advanced to the 8-celled stage or the morula.

It is of interest to note the early disappearance of ova after these injections. After

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[1] The stilbestrol was supplied through the courtesy of Dr. J. A. Morrell of E. R. Squibb & Sons.

TABLE 1. EFFECT OF STILBESTROL ON FERTILIZED OVA

| No. of animal | Injections | Hr. a.v.p. | Hr. after first injection | Number and condition of ova | |
|---|---|---|---|---|---|
| | | | | Tubes | Uterus |
| | | | | *Injections of 1.0 mg. Stilbestrol* | |
| 5 | 1/k | 32 | 21 | 6 ova; blastomeres indefinite; 3 2-celled, flattened, yellowish. | |
| 8 | 1/k | 36 | 28 | 5, 2-celled. | |
| 6 | 1/1/k | 53 | 45 | | 1 from vagina; 1 empty zona from lower uterus or vagina. |
| 22 | 1/1/k | 54 | 45 | 4 from intramural. 1 other from intramural, about 5 cells beginning to fragment. | |
| 7 | 1/1/k | 55 | 47 | | |
| 23 | 1/1/k | 55 | 48 | | |
| 4 | 1/–/k | 60 | 49 | | 4 1-celled; yellow and swollen, fine granules. |
| 10 | 1/1/k | 62 | 54 | | 2 1-celled; 1 2-celled, fragmenting. |
| 20 | 1/1/1/k | 78 | 69 | | |
| 1 | 1/–/1/k | 82 | 74 | 1 ovum. | |
| 3 | 1/1/1/k | 83 | 70 | | |
| 2 | 1/–/1/1/k | 96 | 88 | | |
| | | | | *Injections of 0.5 mg. Stilbestrol* | |
| 16 | .5/.5/k | 49 | 36 | | |
| 14 | .5/.5/k | 55 | 42 | | |
| 17 | .5/.5/k | 60 | 47 | 3 ova; 2 about 8-celled. | |
| 15 | .5/.5/k | 62 | 49 | 3 ova; 8- to 10-celled. | |
| | | | | *Injections of 0.005 mg. Stilbestrol. x=0.005 mg.* | |
| 26 | x/x/k | 60 | 54 | 4 ova; 1 about 12-celled; 1 in morula stage. | |
| 39 | x/x/x/k | 78 | 70 | | 1 late morula or collapsed blastocyst. |
| 25 | x/x/x/k | 79 | 71 | 1 in lower tube, clear zona, very pale yellow. | |
| 38 | x/x/x/k | 80 | 72 | | 1 irregular in shape, possibly blastocyst, collapsed and yellow. |
| 35 | x/x/x/k | 83 | 71 | | |
| 33 | x/x/x/x/k | 102 | 95 | 1 late morula with zona. | |
| 24 | x/x/x/x/k | 102 | 89 | 3 collapsed blastocysts. | |
| 34 | x/x/x/x/k | 104 | 97 | 1 good, early blastocyst with zona. | 1 shrunken blastocyst without zona. |
| 37 | x/x/x/–/k | 107 | 95 | 3 degenerate blastocysts, 1 still recognizable with cells compact, no zona. 3–4 blastocysts (?), clear. | |
| 36 | x/x/x/–/k | 108 | 96 | 2 blastocysts (?). | |
| 28 | x/x/–/x/k | 108 | 100 | 3 irregular, yellow blastocysts, no zona in isthmus. 1 clear blastocyst, inner-cell mass not normal; 3 ova. | 1 blastocyst. |
| 27 | x/x/–/x/k | 109 | 101 | 1 morula or contracted blastocyst with zona. | 2 irregular blastocysts without zonas. |

a.v.p.=after finding the vaginal plug.
k=autopsy.

55 hours a.v.p. only 8 ova were seen (1 in tube, 7 in uterus), whereas there was a total of at least 48 corpora lutea in this group of animals.

Our findings on the effects of stilbestrol on the corpora lutea are hard to interpret

because some ovaries showed large, pink corpora lutea as early as 32 hours a.v.p. (mice 5, 8 and 18) after 1 injection; not much enlarged in mice 6, 7 and 22 with 2 injections 50 to 54 hours a.v.p.; white knobby corpora lutea in *mouse 20;* yet the corpora were rather prominent and pink in *mouse 2* at 96 hours a.v.p.

*Effects of daily injections of 0.5 mg. stilbestrol.* This group was too small for definite conclusions, but the dosage was somewhat less toxic than 1 mg. injections. Eggs in the oviducts of 2 of the 4 animals had advanced to the 8- and 10-celled stages, whereas after the 1 mg. injections embryos had not advanced beyond the 2-celled stage.

*Effects of daily injections of 0.005 mg. stilbestrol.* This reduced dosage of stilbestrol, 1/200th less than that in the first series, was sufficient to disrupt the reproductive process in the mouse. Some of the ova survived to develop into blastocysts, all but one of which showed signs of degeneration. However, only 25 blastocysts or objects resembling blastocysts could be found after 70 hours a.v.p., and yet at least 80 corpora lutea were counted. Furthermore, of these 25 blastocysts, all but 2 were found in the tubes, 70 to 109 hours a.v.p.

Since most of the autopsies in this series were delayed until after the fourth day a.v.p., to ascertain the effect on tubal passage of ova, transitory changes in the ovaries were not seen. However, in mice 26 and 25, autopsied 60 to 79 hours a.v.p., there were pinkish corpora lutea, but in all of the animals (except *mouse* 36) autopsied 80 hours a.v.p., the corpora were white, although still prominent. These were doubtless undergoing regression.

## DISCUSSION

It is fairly difficult to compare, in terms of units, the effects of stilbestrol with other estrogens on the passage of ova through the oviducts, because many of the ova disintegrate after both small and large doses of stilbestrol. However, there is sufficient evidence to show that acceleration may be brought about as readily after 1 mg. injection of stilbestrol as with 200 R.U. of progynon-B (Schering); and that retention of the blastocysts within the tubes is as common following daily injections of 0.005 mg. stilbestrol as with 10 R.U. of theelin (Parke, Davis) and amniotin (Squibb). This is in line with the work of Dodds, Golberg, Lawson and Robinson (4), who found stilbestrol to be 2.5 times as active as estrone and at least as active as estradiol.

Although the tubal reactions following these estrogens are similar, the apparent more toxic action of stilbestrol on the ova should be considered. We have no way of knowing whether this action is direct or indirect, but are inclined to believe that the effects are more direct than merely a change of tubal fluid density, which we thought probable when using 200 R.U. progynon-B (3). Selye (5) found that stilbestrol is much more toxic in mice than equal doses of other estrogens. With a total of 13 mg. injected over 14 days, 4 of his 6 animals died from liver damage or acute hemorrhagic pneumonia.

Stilbestrol also has a deleterious effect on the corpora lutea of early pregnancy, especially with the 0.005 mg. dosages. The corpora lutea begin to regress (whiten) approximately 80 hours a.v.p. and by 100 hours are as white as the corpora lutea following injections of testosterone propionate (6). This is probably due to the cessation of LH secretion.

Furthermore, the injections of 1 mg. stilbestrol did not result in the large corpora lutea usually seen after massive injections of other estrogens. The repeated daily injections in these experiments may have resulted in an impaired pituitary, because Noble (7) has reported this condition following stilbestrol injections.

As a result of these experiments, it is apparent that the synthetic estrogen, stilbestrol, has, in common with other estrogens, the capacity to modify the normal de-

velopment, tubal passage, and implantation of mouse embryos. This corroborates the findings of Parkes, Dodds and Noble (1), but carries their work a little further to show something of the history of the eggs subsequent to the injections. Interruption of pregnancy is the end result with both the 0.005 mg. and 1 mg. injections, but it is not necessary to use the larger dosage of stilbestrol, and is unwise because of possible toxicity.

## SUMMARY

The synthetic estrogen, stilbestrol, has, in common with other estrogens, the capacity to modify the passage of fertilized ova through the oviducts of mice.

Daily subcutaneous injections of 1.0 mg. stilbestrol dissolved in sesame oil cause an acceleration of the rate of tubal passage, while injections of 0.005 mg. result in the tube-locking of the ova. Pregnancy is interrupted with both dosages. Use of the larger amount is not only unnecessary but unwise because of possible toxic effects in other vital areas.

Stilbestrol apparently has a toxic effect on the ova. It also has a deleterious effect upon the corpora lutea of early pregnancy, causing them to begin regression (blanching) on the fourth day.

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# RATE OF ABSORPTION OF ANDROGENS AND ESTROGENS IN FREE AND ESTERIFIED FORM FROM SUBCUTANEOUSLY IMPLANTED TABLETS

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THE TECHNIQUE of administering crystalline hormones by the subcutaneous implantation of crystals or compressed tablets has now been extensively investigated. This method, introduced by Deanesly and Parkes (1) is of particular use when a prolonged, uninterrupted action is desired. Its effectiveness and economical use of the active substance has been fully described elsewhere, and the recent communication by Warwick and Parkes (2) may be consulted for a short survey of the relevant literature.

It is now apparent that the steroid hormones (3) and some of the synthetic estrogens (4) may be implanted in tablet form into man without causing untoward local reactions, and with considerable clinical success. To quote Geist et al. "The local tissue response to implanted crystals and pellets is a typical non-specific foreign body reaction. Following the implantation of crystals of $\alpha$-estradiol and $\alpha$-estradiol benzoate, the tissue surrounding the crystals responds by the formation of tiny nodules. In contrast to this type of reaction, a thick avascular capsule forms about pellets of the implanted hormone. It is important to note that the epidermis overlying the implants showed no evidence of cellular atypism and, similarly, in no instance did the subcutaneous tissues in contact with, and adjacent to, the implanted hormone show any abnormal cellular proliferation." There are two outstanding problems which reman in connection with the technique, namely, control of the dosage and of its rate of change.

In rats, testosterone tablets weighing between 1.0 and 2.5 mg. were found by Deanesly and Parkes (1) to lose 60 to 75% of their weight in 10 days, and testosterone propionate tablets lost 20 to 25% during the same period. Other androgens were absorbed at lower rates. In the guinea pig, testosterone tablets of 29 and 100 mg. lost 25 and 24% per month respectively, and the propionate 17% per month from 20 mg. tablets (5).

Tablets of estrone in rats lose on the average between 2.5 and 6% per month of 30 days (6) more or less independently of their initial weight, when this is between 6 and 60 mg. In guinea pigs and mice the rate of absorption appears to be considerably higher, up to 9% per month (5, 6) Estradiol is absorbed from tablets at more than twice the rate of estrone (6). Lipschütz and Vargas (7) demonstrated that the rate of absorption of estradiol from tablets implanted into guinea pigs is dependent on the weight and shape of the tablets, and might vary between 4 and 40% per month, being lower the heavier the tablet.

Progesterone tables weighing ca. 50 mg. were found by Warwick and Parkes (2) to lose about 20% per month when implanted into goats, guinea pigs or rats (range 12–27%) and desoxycorticosterone acetate in rats lost 14 to 30% per month from 36 to 54 mg. tablets in rats, while free desoxycorticosterone was absorbed at more than twice this rate.

It is clear that the amount of material lost by a tablet of any given substance is not only dependent on its solubility in body fluids, but may be affected by its state of compression, the site of implantation, the species of animal used, and by the shape

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of the tablet. Whatever the mechanism of absorption, the surface area of the tablet will, other things being equal determine the rate of absorption. In a well-made product, the surface is hard and smooth, and consequently may be less readily eroded than that of a lightly-compressed or rough-surfaced tablet. The figures in the literature have often been compiled from very heterogeneous material, hence the large variation in the rate of absorption from tablets of equal weight of the same substance. For tablets of any given shape, the initial rate of absorption should theoretically be proportional to $W^{2/3}$, where $W$ is the weight, but the subsequent rate will depend on the actual shape and behavior of the tablet. A sphere would, if it remained spherical, continue to show the same relationship between its weight and the rate of absorption, but a flat disc would give a much more uniform rate, since the thickness of the disc would diminish rapidly in proportion to its diameter. If the erosion of the surface is irregular the surface area may actually increase and absorption be hastened as the tablet ages, although its weight is becoming less.

In general, esterification of the steroid hormones decreases their availability and prolongs their action by injection. This is also seen when dealing with tablets, and esterification offers the most simple means of regulating dosage, if the rate of absorption from tablets of the free hormone is too high. As will be seen later, it is possible to select an appropriate aliphatic monoester or diester which will give, with fair accuracy, almost any desired daily dosage of hormone.

The present paper is concerned with the two problems discussed above. The rate of absorption of a number of esters of testosterone, estradiol and diethylstilbestrol has been compared with that of the parent substances under as uniform conditions as could be arranged, and has been followed for a sufficient length of time for changes in it as the tablet is absorbed to become clear.

## MATERIAL AND METHODS

The tablets were disc-shaped and of as uniform a shape and weight as was practicable, and were made in the laboratory with a hand-press. They were usually very near to 40 mg. in weight and always between 36 and 42.5 mg. They were heavily compressed to a diameter of 6 mm. and a central thickness of 2.1 to 2.3 mm., thinning towards the edges to about 1.2 mm., and must be distinguished from lightly compressed tablets of the 'hypodermic' type, which are designed to disintegrate rapidly on contact with fluid.

Up to four tablets of each substance were implanted subcutaneously into adult male rats, one per rat. The operation was performed under avertin anesthesia, the tablets pushed well under the skin of the flank with forceps and the incision closed by one or two stitches. The tablets were then removed at intervals and reimplanted into a fresh site after being cleaned, dried and weighed. Very occasionally a tablet was lost by extrusion from the site of implantation, or by death of the host. In certain other cases, indicated in the tables, disintegration of the tablet precluded a determination of its weight.

*Testosterone and its derivatives.* Table 1 and figure 1 show the results obtained with a series of androgens of the testosterone group. The rates of absorption vary from 36.8% per month of 30 days for free testosterone to 0.23% per month or less for testosterone benzoate, which remains virtually unabsorbed. The percentages of the free hormone given per month by each ester are shown in brackets in the last column of table 1.

Methyl testosterone was absorbed at about the same rate as testosterone while the tablets remained intact, but they did not survive for a long period without disintegrating. Testosterone hydroxylamine-acetate and testosterone propionate hydrox-

TABLE 1. RATE OF ABSORPTION OF TESTOSTERONE AND VARIOUS DERIVATIVES AND ESTERS, IMPLANTED SUBCUTANEOUSLY IN TABLET FORM IN MALE RATS

| Substance. (T = testosterone) | Initial wt. of tablet | Wt. of tablet after (days) | | | | % absorbed per month.[1] ( ) = free hormone. |
|---|---|---|---|---|---|---|
| | | 34 | 66 | 91 | 171 | |
| | *mg.* | | | | | |
| Free T. | 40 | 23.5 | 9 | 0 | — | |
| | 40 | 20.5 | 6.5 | 0 | — | |
| | 41 | 20.5 | 7 | 0 | — | 36.8 |
| | 40 | 21.5 | 7.5 | 0 | — | |
| Mean | 40.3 | 21.5 | 7.5 | 0 | — | |
| Methyl T. | 42 | Tablets disintegrating | — | — | — | — |
| | 40 | | — | — | — | |
| | 40.5 | 22 | — | — | — | |
| | 40.5 | 26.5 | <12 (disint.) | — | — | |
| T. propionate | 40.5 | 28 | 19 | 13 | 0 | 21.8 (18.8) |
| | 40 | 28 | 18 | 13.5 | 0 | |
| | 40 | 28.5 | 19 | 14 | 0 | |
| | 40 | 28 | 19 | 14 | 0 | |
| Mean | 40.1 | 28.1 | 18.8 | 13.6 | 0 | |
| T. 3-acetate-17-n-butyrate | 40 | 37.5 | — | 29.5 | 21.5 | 8.2 (6.1) |
| | 40 | 37 | — | 30 | 21 | |
| | 42 | 37 | — | 31 | 22.5 | |
| Mean | 40.7 | 37.2 | — | 30.2 | 21.7 | |
| T. 3-acetate-17-propionate | 42 | 39.5 | — | 35 | — | 6.2 (4.8) |
| | 42.5 | 40 | — | 36 | 30 | |
| | 40.5 | 37 | — | 32 | 25.5 | |
| | 41 | 38.5 | — | 32.5 | 25 | |
| Mean | 41.5 | 38.7 | — | 33.9 | (26.8)[2] | |
| T. dipropionate | 41 | 39 | — | 36 | 29 | 5.2 (3.9) |
| | 41.5 | 40 | — | 36 | 29.5 | |
| | 40 | 37.5 | — | 34 | 26.5 | |
| | 41.5 | 39 | — | 35.5 | 30 | |
| Mean | 41.0 | 38.9 | — | 35.4 | 28.8 | |
| T. propionate oxime | 41.5 | 40 | — | 36.5 | 32.5 | 3.8 |
| T. benzoate | 38 | 38 | — | 37 | split | ca. 0.23 |
| | 38 | 38.5 | — | 38.5 | 38 | |
| | 41 | 40 | — | 40 | 38.5 | |
| | 40.5 | 40.5 | — | 40.5 | 40.5 | |
| Mean | 39.4 | 39.3 | — | 39.0 | (39.3)[2] | |
| T. hydroxylamine acetate | 40 | 0 | — | — | — | — |
| T. propionate hydroxylamine acetate | 36 | 0 | — | — | — | — |
| | 39 | 0 | | | | |

[1] 30 Days.
[2] Means in brackets indicate a change in the initial mean weight due to loss of a tablet.

ylamine-acetate tablets also apparently disintegrated rapidly, more rapidly than those of methyl testosterone, and were completely absorbed within the first 34 days. As no trace of these substances was found at the site of implantation it is possible that they were, in fact, very rapidly absorbed without disintegration of the tablet, but this seems improbable in view of their constitution. No further supplies were available for a repetition of the work. It appears, therefore, that care must be taken before using an androgen in tablet form to ascertain whether it is liable to disintegrate and to submit the host to a sudden large overdose.

Variation in percentage loss from tablet to tablet of the same substance is reassuringly small, and it seems that the dosage given by a tablet may be predicted with

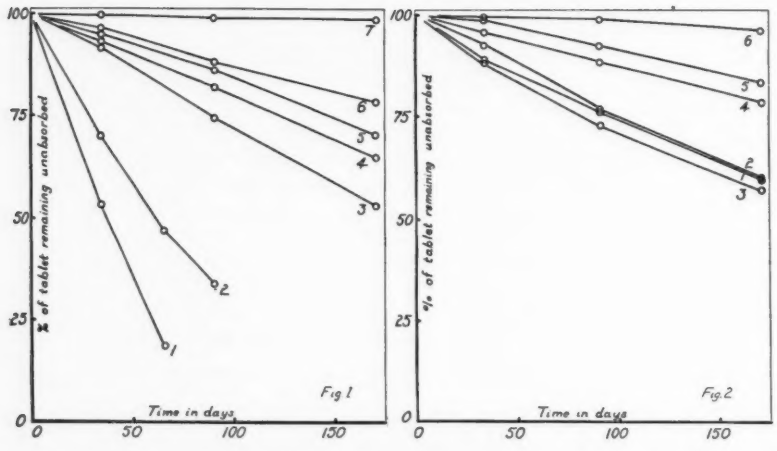


Fig. 1. The absorption of testosterone and its esters (table 1) from tablets of approx. 40 mg. subcutaneously implanted in male rats.

1. Free testosterone (4 tablets), 2. Testosterone propionate (4 tablets), 3. Testosterone-3-acetate-17-*n*-butyrate (3 tablets), 4. Testosterone-3-acetate-17-propionate (4 tablets), 5. Testosterone dipropionate (4 tablets), 6. Testosterone propionate oxime (1 tablet), 7. Testosterone benzoate (4 tablets).

Fig. 2. The absorption of estriol, and of estradiol and its esters (table 2) from tablets of approx. 40 mg. subcutaneously implanted into male rats.

1. Estriol (3 tablets), 2. Estradiol (4 tablets), 3. Estradiol dipropionate (3 tablets), 4. Estradiol-17-propionate (3 tablets), 5. Estradiol-17-caprylate (1 tablet), 6. Estradiol-3-benzoate (4 tablets).

little error under these conditions. The figures for the benzoate are unreliable, as insufficient material was lost for adequate estimation of the rate of absorption, which is clearly very low.

*Naturally occurring estrogens.* The data for these substances are given in table 2 and illustrated by figure 2. Estriol and estradiol are both absorbed at a rate of 7 per cent per month, one of three tablets of estriol disintegrating after several months. The rate of absorption agrees with Deanesly's (6) figures, and would appear to be about twice that of estrone. Equilenine tablets disintegrated and were present as a sludge 34 days after implantation.

The two aliphatic monoesters of estradiol agree with the testosterone series in showing decreasing rates of absorption as the carbon chain lengthens, and the benzoate is again only very slowly attacked. Estradiol dipropionate, however, was absorbed rather more rapidly than free estradiol. The explanation of this is obscure. Once more, variation in the rate of absorption from similar tablets is low.

With these and the foregoing androgens, the rate of absorption changes very little with time, even when more than half of the tablet has gone. Theoretically, as the surface area of such a tablet decreases continually, the absorption of material should fall. The surfaces of the more readily absorbed tablets tend, however, to become a little roughened and to loosen somewhat, so that absorption becomes facilitated as the tablet ages, and the expected fall in the rate of absorption may not occur

TABLE 2. RATE OF ABSORPTION OF SOME NATURALLY OCCURRING ESTROGENS AND ESTERS OF ESTRADIOL, IMPLANTED SUBCUTANEOUSLY IN TABLET FORM IN MALE RATS

| Substance | Initial wt. of tablet | Wt. of tablet after (days) | | | % absorbed per month[1] ( )=free hormone |
|---|---|---|---|---|---|
| | | 34 | 91 | 171 | |
| | mg. | | | | |
| Estriol | 40 | 35 | 30 | 23 | 7.0 |
| | 41.5 | 37.5 | 32.5 | disintegrated | |
| | 40 | 35.5 | 30 | 25 | |
| Mean | 40.5 | 36.0 | 30.8 | (24.0)[2] | |
| Equilenine | 40 | tablets disintegrated | — | — | — |
| | 40 | | — | — | |
| Estradiol | 40.5 | 38 | 33 | 25.5 | 7.0 |
| | 39 | 36 | 30 | 24 | |
| | 40 | 36 | 29.5 | 23.5 | |
| | 40 | 37.5 | 30 | 23 | |
| Mean | 39.9 | 36.9 | 30.6 | 24.0 | |
| Estradiol dipropionate | 39 | 34 | 27.5 | — | 7.5 (4.5) |
| | 40 | 35.5 | 30 | 24 | |
| | 40.5 | 35.5 | 29.5 | 22 | |
| Mean | 39.8 | 35.0 | 29.0 | (23.0)[2] | |
| Estradiol-17-propionate | 41 | 39 | 36 | 32 | 3.8 (3.3) |
| | 39 | 37.5 | 34.5 | 30.5 | |
| | 40 | 38 | — | — | |
| Mean | 40.0 | 38.2 | 35.3 | 31.3 | |
| Estradiol-17-caprylate | 39.5 | 39 | 36.5 | 33 | 2.9 (2.0) |
| Estradiol-3-benzoate | 41 | 40.5 | 40 | — | ca. 0.65 |
| | 40 | 39.5 | 39.5 | 38 | |
| | 40 | 40.5 | 40.5 | 39 | |
| | 40 | 39.5 | 39.5 | — | |
| Mean | 40.3 | 40.0 | 39.9 | (38.5)[2] | |

[1] 30 days.
[2] Means in brackets indicate a change in the initial mean weight due to loss of a tablet.

until considerably later. It is also conceivable that the tissues around the tablet become better adapted to attacking it, but as the site is changed from time to time this cannot be a factor of great importance. Tablets which disintegrate show this tendency in an extreme way, and those, such as the benzoates and higher aliphatic esters, which are very slowly absorbed, do not lose sufficient material within the test period for a slowing of the absorption rate to be observed, even if it occurs. One theoretic drawback to the use of tablets—a continuously decreasing dosage level—is thus found in practice to be of negligible importance.

TABLE 3. RATE OF ABSORPTION OF DIETHYLSTILBESTROL AND ITS DIESTERS FROM TABLETS IMPLANTED SUBCUTANEOUSLY IN MALE RATS

| Substance. Diethylstilbestrol | Initial wt. of tablet | Wt. of tablet after (days) | | | | % absorbed per month[3] ( )=free hormone |
|---|---|---|---|---|---|---|
| | | 38 | 105 | 190 | 275 | |
| | mg. | | | | | |
| Free (1st group) | 39 | ca. 3 | — | — | — | — |
| | 40 | 0 | — | — | — | |
| | 42 | 0 | — | — | — | |
| Free (2nd group) | 40 | 29.5 | 0 | — | — | (19.2) |
| | 40 | 29 | 0 | — | — | |
| | 40 | 28.5 (43 days) | 0 (91 days) | — | — | |
| Diacetate | 40 | 32 | 20 | ca. 10 | — | 12.0 (9.9) |
| | 36 | 33.5 | 25.5 | 16 | — | |
| | 40 | 36.5 | 22.5 | — | — | |
| | 40 | 37 | 23 | 15 | — | |
| Mean | 39.0 | 34.8 | 22.8 | (13.7)[1] | — | |
| Dipropionate | 40 | 35 | — | — | — | 9.7 (7.3) |
| | 40 | 37 | 27.5 | disint.[2] | — | |
| | 40 | 37.5 | 27.5 | 15.5 | 6.5 | |
| | 40 | 36 | 22 | 10.5 | 2 | |
| Mean | 40.0 | 36.4 | 25.7 | 13.0 | 4.3 | |
| Di-*iso*-butyrate | 40 | 39.5 | 33 | 21 | 11 | 6.8 (4.9) |
| | 40 | 37.5 | 36 | 19.5 | 10.5 | |
| | 40 | 40.5 | 34 | 25 | 14 | |
| | 40 | 38 | 32 | 23 | 16 | |
| Mean | 40.0 | 38.9 | 33.8 | 22.1 | 12.9 | |
| Di-*n*-butyrate | 40 | 40 | 36.5 | — | — | 3.5 (2.5) |
| | 40 | 39.5 | 38 | 31.5 | 27 | |
| | 40 | 39.5 | 38 | 32.5 | 27 | |
| | 40 | 39 | 37 | 32 | 27 | |
| Mean | 40.0 | 39.5 | 37.4 | 32.0 | 27.0 | |
| Di-*n*-valerate | 42 | 42 | 40 | disint. | — | 3.4 (2.2) |
| | 40 | 40.5 | 34.5 | 34 | 28.5 | |
| | 40 | 39.5 | 37.5 | 35 | 26.5 | |
| | 40 | 38.5 | 36 | — | — | |
| Mean | 40.5 | 40.1 | 37.0 | (34.5) | (27.5) | |
| Dicaproate | 38 | 36 | 35 | disint. | disint. | 2.7 (1.6) |
| | 40 | 40 | 38.5 | 35 | 30 | |
| | 40 | 40 | 37 | 34 | disint. | |
| | 40 | 40 | 39 | 37 | | |
| Mean | 39.5 | 39.0 | 37.4 | (35.3) | (30.0) | |
| Dilaurate | 40 | 40 | 39 | 38 | 38 | 2.0 (0.88) |
| | 36 | 34 | 30.5 | 27 | 24 | |
| | 40 | 41 | 39 | — | — | |
| Mean | 38.7 | 38.3 | 36.2 | (32.5) | (31.0) | |
| Dipalmitate | 37 | 35 | 34 | 33 | 30.5 | 1.3 (0.48) |
| | 40 | 41 | 39.5 | 37 | disint. | |
| | 40 | 41 | 40.5 | 40 | 37 | |
| | 40 | 39 | 38 | 37 | disint. | |
| Mean | 39.3 | 39.0 | 38.0 | 36.7 | 33.8 | |

TABLE 3—*Continued*

| Substance. Diethylstilbestrol | Initial wt. of tablet | Wt. of tablet after (days) | | | | % absorbed per month[3] ( )=free hormone |
|---|---|---|---|---|---|---|
| | | 38 | 105 | 190 | 275 | |
| | mg. | | | | | |
| Dibenzoate | 40 | 39.5 | 39 | 39 | 39 | |
| | 40 | 39.5 | 38.5 | 39 | 39 | ca. 0.01 |
| | 37 | 38 | 38 | 38.5 | 38 | |
| | 36.5 | 36.5 | 36 | 36.5 | 36.5 | |
| Mean | 38.4 | 38.4 | 37.9 | 38.3 | 38.1 | |

[1] Means in brackets indicate a change in the initial mean weight due to loss of a tablet.
[2] disint.=disintegrated.
[3] 30 days.

Geist et al. (3) conclude, from their observations on the therapeutic and physiological effects of tablets of estradiol implanted subcutaneously in women, that a tablet, which is eventually surrounded by a thick capsule, ceases to be absorbed in appreciable amounts. This stoppage of absorption occurs after a period of about three months, and is not seen with crystals of the same substance, which do not become heavily encapsulated. Tough, fibrous capsules are frequently formed in the rat, but do not appear to have interfered with absorption in the present series of experiments, in which tablets have remained in situ for three months before reimplantation at a new site.

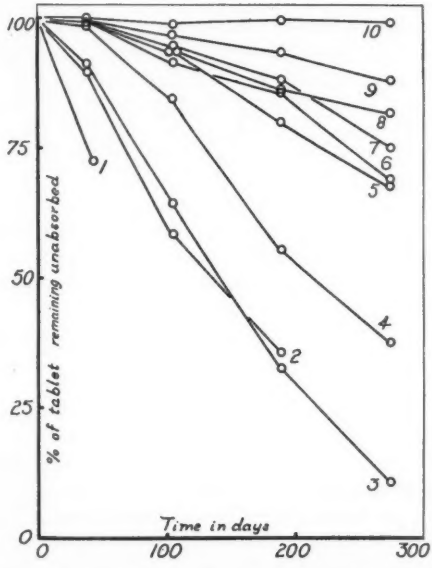


Fig. 3. THE ABSORPTION OF DIETHYLSTILBESTROL AND ITS DIESTERS (TABLE 3) FROM TABLETS OF APPROX. 40 MG. SUBCUTANEOUSLY IMPLANTED INTO MALE RATS.

1. Free diethylstilbestrol (3 tablets), 2. Diethylstilbestrol diacetate (4 tablets), 3. Diethylstilbestrol dipropionate (4 tablets), 4. Diethylstilbestrol di-*iso*-butyrate (4 tablets), 5. Diethylstilbestrol di-*n*-butyrate (4 tablets), 6. Diethylstilbestrol divalerate (4 tablets), 7. Diethylstilbestrol dicaproate (4 tablets), 8. Diethylstilbestrol dilaurate (4 tablets), 9. Diethylstilbestrol dipalmitate (4 tablets), 10. Diethylstilbestrol dibenzoate (4 tablets).

*Diethylstilbestrol and its diesters.* A large series of the aliphatic diesters of diethylstilbestrol has been investigated, as this relatively inexpensive synthetic estrogen is rapidly absorbed from tablets, and control is more probably needed in the direction of decreasing the rate of absorption than with the natural estrogens.

Free diethylstilbestrol disintegrates rapidly and regularly in tablets of the type dealt with in our experiments. As these tablets are hard and compact, a tablet upon which reliance could be placed would, it seems, have to be very highly compressed. Of an initial group of 3 tablets, 2 had disappeared within 38 days, and the remains of the third weighed about 3 mg. A second group of 3 tablets was intact after 43 days, but disintegrated shortly afterwards and was completely absorbed by 91 days. While it remains in tablet form stilbestrol is absorbed at a rate of about 19 per cent per month (table 3, fig. 3).

The diesters show a regularly decreasing rate of absorption as the aliphatic chain lengthens, and the iso-butyrate is more rapidly absorbed than the n-butyrate. Once more, the dibenzoate is very slowly absorbed.

The rates of absorption of this series are less regular, both from tablet to tablet and substance to substance, than with the natural estrogens and the testosterone series. At the same time, the initial rate is regularly slower than that attained after a month or two of implantation. Both of these observations can be explained by the irregular surface which tends to develop on the tablets, to a markedly greater extent with most of the other substances investigated. Eventually, tablets of even the higher esters of diethylstilbestrol tend to crumble, as may be seen from table 3, but this did not occur until after 4 or more months of implantation, nor in the majority of tablets. It is possible that a tendency to crumble in this manner is bound up with the original state of the material from which the tablets were made. Diethylstilbestrol and its esters were in a very finely divided state, whereas most of the other compounds were originally in the form of recognizable small crystals.

The absorption rates given in table 3 are thus more of a series of averages than in the other tables, and do not represent a level which can be relied upon from the start. Very high compression might overcome this difficulty, but it would probably result in slower rates of absorption than those quoted.

These drawbacks excepted, the series of diesters gives a wide choice of dosage, and any desired dose level could be selected. It is unfortunate that, as far as the evidence now presented for the stilbestrol series is concerned, as high a degree of reliability cannot be attached to such a predicted output as appears justified in other instances. Progress in the making of homogeneous and stable tablets should solve this problem.

## DISCUSSION

In view of the variations which might be anticipated in the vascularity, lymphatic supply, etc., of an area surrounding an implanted tablet, the rates of absorption of similar tablets implanted into different animals remain surprisingly regular. With the naturally occurring estrogens and testosterone and their esters, a variation of as high as $\pm 10\%$ from the mean is exceptional. Even with the diethylstilbestrol diesters, nearly all of the individual determinations are within $\pm 25\%$ of their mean when sufficient absorption has taken place for accurate estimation. These high levels of consistency are considerably better than those previously reported in the literature, and must be attributed to the use of a standard experimental technique.

The dose given by a tablet may therefore be relied upon within fairly narrow limits, and this dose is furthermore known to be absorbed by the host. It is usually assumed that an injection in, say, an oil solution, is more reliable as to dosage than

TABLE 4. THE THEORETICAL RATE OF ABSORPTION (I.E. OUTPUT OF MATERIAL) FROM A DISC WITH A THICKNESS OF 2 MM. AND A DIAMETER OF 6 MM. AND FROM A CYLINDER OF LENGTH 18 MM. AND DIAMETER OF 2 MM., ASSUMED TO BE MADE OF THE SAME EQUALLY COMPRESSED MATERIAL. THESE TABLETS WOULD BE OF EQUAL INITIAL WEIGHT, AND BOTH COMPLETELY ABSORBED AT TIME 100.

| % of total lifetime consumed | Disc | | | | Cylinder | | | |
|---|---|---|---|---|---|---|---|---|
| | Diameter | Thickness | Relative weight (volume) | Relative absorption rate | Diameter | Length | Relative weight (volume) | Relative absorption rate |
| | *mm.* | *mm.* | | | *mm.* | *mm.* | | |
| 0 | 6.0 | 2.0 | 576 | 240 | 2.0 | 18.0 | 576 | 304 |
| 25 | 5.5 | 1.5 | 363 | 187 | 1.5 | 17.5 | 315 | 219 |
| 50 | 5.0 | 1.0 | 200 | 140 | 1.0 | 17.0 | 136 | 140 |
| 75 | 4.5 | 0.5 | 81 | 99 | 0.5 | 16.5 | 33 | 67 |
| 100 | 4.0 | 0.0 | 0 | 64 | 0.0 | 16.0 | 0 | 0 |

absorption from a tablet, but this must be seriously questioned. Such an injection may, according to the locality in which it comes to rest, be rapidly or slowly absorbed and subject to even more vicissitudes than an implanted tablet. As with a tablet, one may assume that, if left long enough, it will all be absorbed and utilized or excreted, but the rate of absorption may be no more predictable in one case than in the other.

Convenience of implantation has led to the adoption by some clinicians of cylindrical tablets rather than disc-shaped ones. It is interesting to compare the theoretical rates of absorption to be expected from such tablets. A disc having a thickness equal to one-third of its diameter, say, 6 mm. by 2 mm., approximately like the tablets used in this work, will weigh the same as a cylinder with a length of 18 mm. and a diameter of 2 mm. The initial rate of absorption from these two solids, supposedly made of the same substance under the same degree of compression, will not be the same, since the surface area of the cylinder exceeds that of the disc. However, they will take the same time to be completely absorbed, since an erosion to a depth of 1 mm. from the surface of each will entirely consume it, and half-way through this time

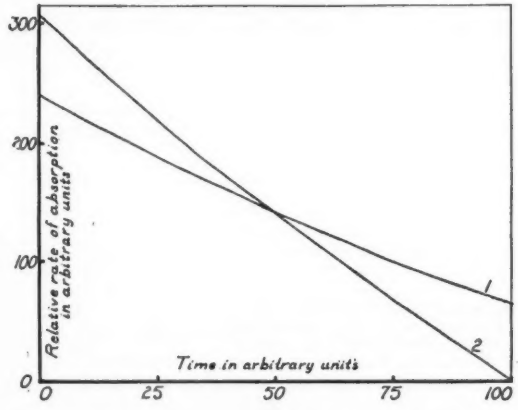


Fig. 4. THE THEORETICAL RATES OF ABSORPTION THROUGHOUT THE LIFE HISTORY OF (1) A DISC-SHAPED TABLET MEASURING 6 MM. IN DIAMETER AND 2 MM. IN THICKNESS, AND (2) A CYLINDRICAL TABLET OF THE SAME INITIAL WEIGHT MEASURING 18 MM. IN LENGTH AND 2 MM. IN DIAMETER. BOTH TABLETS ARE COMPLETELY ABSORBED AT TIME 100. (SEE TABLE 4 AND TEXT.)

they will be undergoing absorption at the same rate. Table 4 shows the linear dimensions, relative weights, and relative absorption rates of the two, initially and at ¼, ½ and ¾ of the total time taken to absorb them. The disc-shaped tablet theoretically gives 58% of the initial output at ½ its lifetime, and still gives 41% at ¾ of that period, whereas the cylinder has dropped to an output of 46% at the half-way point, and to 22% when it is ¾ of the way through its existence. Moreover, at the very end of its life, the disc is giving 27% of the initial dosage, but the dosage yielded by the cylinder drops to zero (fig. 4).

On theoretical grounds, therefore, a flat disc-shaped tablet is to be preferred, when the dose level is to be maintained as high as possible. In practice, we have found that such a disc gives an even higher level of dosage towards the end of its life than is to be expected, due to the irregularities which develop on its surface. It is, of course, theoretically possible with a thin enough tablet to keep the rate of absorption almost constant up to the end, a result which would apparently be gained in practice by tablets with a fair thickness in comparison with their diameter.

## SUMMARY

The absorption from subcutaneously implanted disc-shaped tablets of various estrogens and androgens and their esters has been studied in male rats.

Tablets of testosterone and its esters, of estriol, and of estradiol and its esters are absorbed at a remarkably steady rate, and do not show such a decrease in absorption as should theoretically occur. The variation in the rate of absorption from tablet to tablet of the same substance is small.

Esterification decreases the rate of absorption, with the unexplained exception of estradiol dipropionate, which was absorbed rather more rapidly than free estradiol. A wide range of dosage is thus available.

Diethylstilbestrol and its diesters are less regularly absorbed from tablets than the foregoing substances, but the variation is still not excessive. Tablets of the diethylstilbestrol series tend to exhibit increased rates of absorption as time goes on. This may be explained by the irregularities which develop on their surfaces.

Occasional tablets of the diethylstilbestrol diesters and all tablets of diethylstilbestrol itself disintegrated during the experiments. This was also seen with methyl testosterone, equilenine, and with one of three tablets of estriol. The danger of such sudden disintegration might be avoided by the use of very highly compressed tablets, but those used were quite hard and smooth at the outset.

I am indebted for the provision of the various free and esterified substances used in this investigation to British Drug Houses Ltd., Organon Laboratories, Parke, Davis & Co., Ltd., Roussel Laboratories and The Schering Corporation, N. Y.

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# THE CHICK COMB RESPONSE TO ANDROGENS

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THIS STUDY was undertaken to determine the quantitative response of the chick's comb to androgens. It was reported by Ruzicka (1) and confirmed by Burrows, Byerly, and Evans (2) that chicks grow combs when injected with androgens. Quantitative response was subsequently claimed by Frank and Klempner in 1937 (3), who applied various androgens in oil to the base of the comb by inunction. They

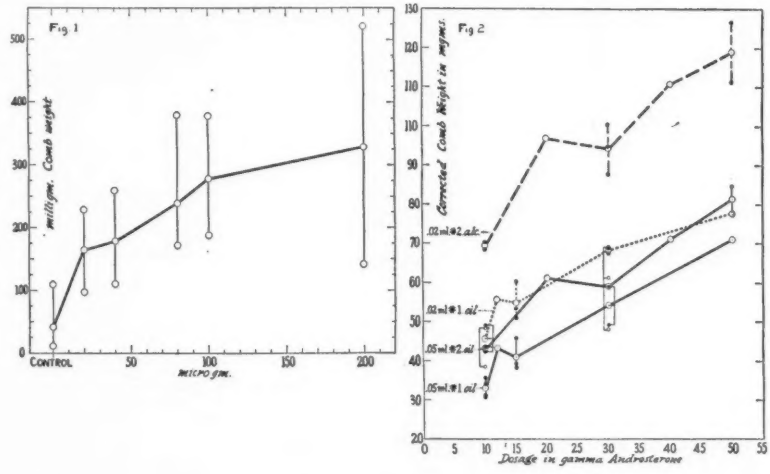


Fig. 1. RESPONSE OF THE CHICK'S COMB TO ANDROSTERONE ACCORDING TO THE DATA OF FRANK AND KLEMPNER (3). FIG. 2. VARIATION IN THE RESPONSE OF THE CHICK'S COMB TO ANDROSTERONE DEPENDING ON THE AMOUNT AND THE NATURE OF THE SOLVENT.

did not present a curve of response in their original report. Dorfman and Greulich (4), who injected known amounts of androgen, presented response curves. We have repeated these experiments, and the two methods will be discussed separately.

While Frank and Klempner present no typical curve of response, they do give some measures of comb growth for various doses of androsterone applied to the chick's comb. We have plotted these values, with the maxima and minima given for the given doses, in figure 1. It will be seen that the variation in response is so large that a quantitative interpretation is impossible. However, Klempner, Frank and Hollander (5, 6) reported in two papers an improvement in technic, which consists

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in changing the volume and the solvent in which the androgen is dissolved. In figure 2 the results from two experiments reported by these workers are charted together. In the first experiment 0.05 cc. of solvent oil was compared with 0.02 cc., and it was shown that the smaller volume produced better results. The next paper reported

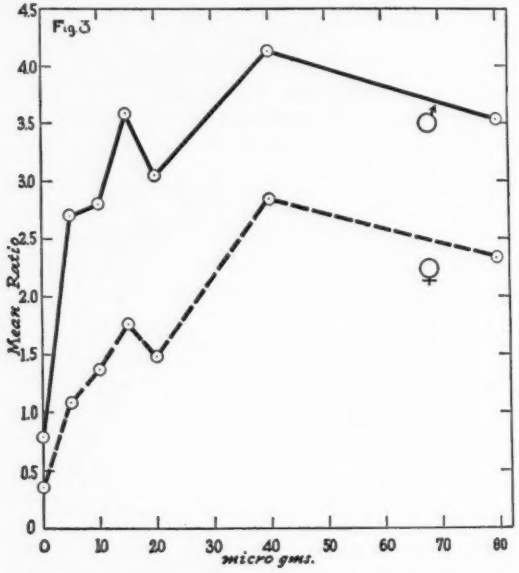


Fig. 3. RESPONSE OF 7-DAY-OLD CHICKS TO ANDROSTERONE (see table 1).

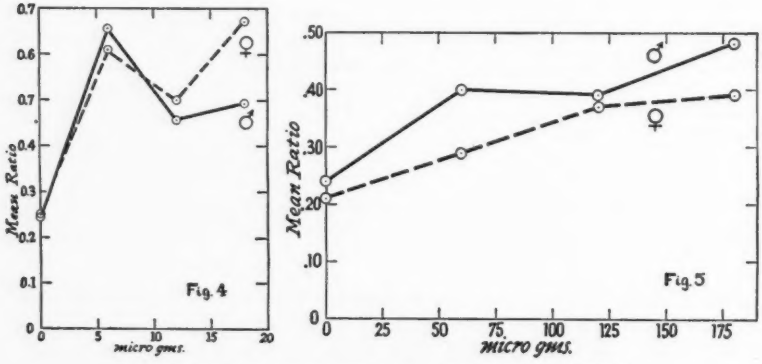


Fig. 4. RESPONSE OF DAY-OLD CHICKS TO ANDROSTERONE (see table 2).
Fig. 5. RESPONSE OF DAY-OLD CHICKS TO ANDROSTERONE (see table 4).

another experiment using 0.05 cc. again, which gave results comparable with the 0.02 cc. of the previous experiment. The graphs of these two experiments together give a good indication of the lack of reliability that this method possesses. In our first experiments 0.1 cc. was used for inunction, but the spreading of the oil on the head made us abandon that amount in favor of 0.05 cc. Even this amount spreads appreci-

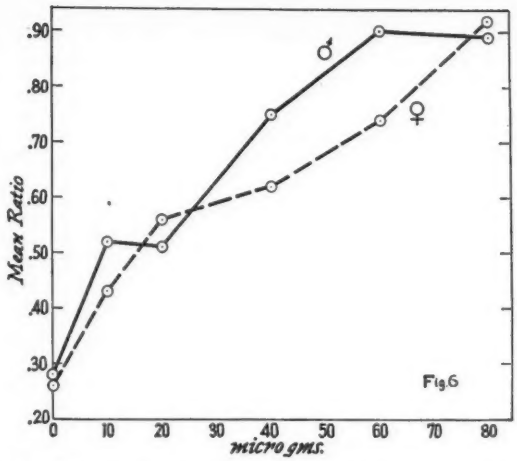


Fig. 6. Response of 4-hour-old chicks to androsterone (see table 5).

Table 1. Response of chick's comb to androsterone applied directly to the comb

| Total dose, γ | No. of animals | Sex | Mean body wt. (B) gm. | Mean comb wt. (C) mg. | Mean ratio C/B |
|---|---|---|---|---|---|
| *A. Ten daily inunctions beginning 7th day after hatching* | | | | | |
| 0 | 6 | M | 125.8 | 99.7 | 0.79 |
| | 3 | F | 113.3 | 40.6 | 0.35 |
| 5 | 2 | M | 119.5 | 323.8 | 2.70 |
| | 4 | F | 117.5 | 128.3 | 1.09 |
| 10 | 3 | M | 119.6 | 335.1 | 2.80 |
| | 3 | F | 125.6 | 171.4 | 1.37 |
| 15 | 3 | M | 128.3 | 460.2 | 3.59 |
| | 3 | F | 124.6 | 219.3 | 1.76 |
| 20 | 4 | M | 124.2 | 379.7 | 3.05 |
| | 2 | F | 104.0 | 155.6 | 1.49 |
| 40 | 2 | M | 132.5 | 546.6 | 4.13 |
| | 4 | F | 123.0 | 350.5 | 2.85 |
| 80 | 5 | M | 125.0 | 442.7 | 3.53 |
| | 1 | F | 140.0 | 327.4 | 2.34 |
| *B. Six daily inunctions beginning 1st day after hatching* | | | | | |
| 0 | 7 | M | 50.2 | 12.3 | 0.25 |
| | 3 | F | 49.9 | 12.6 | 0.25 |
| 6 | 6 | M | 51.4 | 33.9 | 0.66 |
| | 5 | F | 47.7 | 29.4 | 0.62 |
| 12 | 4 | M | 48.6 | 22.4 | 0.46 |
| | 7 | F | 49.0 | 24.7 | 0.50 |
| 18 | 5 | M | 46.6 | 23.0 | 0.49 |
| | 5 | F | 42.7 | 28.8 | 0.68 |

TABLE 2. RESPONSE OF CHICK'S COMB TO ANDROSTERONE, INJECTED SUBCUTANEOUSLY

| Total dose, γ | No. of animals | Sex | Mean body wt. (B) gm. | Mean comb wt. (C) mg. | Mean ratio C/B |
|---|---|---|---|---|---|
| *A. Six days, beginning 7th day after hatching* | | | | | |
| 0 | 5 | M | 90.6 | 66.3 | 0.73 |
| | 7 | F | 78.8 | 27.8 | 0.35 |
| 60 | 5 | M | 85.7 | 62.6 | 0.73 |
| | 7 | F | 83.7 | 41.5 | 0.50 |
| 120 | 7 | M | 92.3 | 94.7 | 1.01 |
| | 3 | F | 88.3 | 38.7 | 0.44 |
| 180 | 2 | M | 95.5 | 144.3 | 1.51 |
| | 9 | F | 90.4 | 67.2 | 0.74 |
| *B. Six days, beginning day after hatching* | | | | | |
| 0 | 12 | M | 51.1 | 12.45 | 0.24 |
| | 9 | F | 51.7 | 10.85 | 0.21 |
| 60 | 5 | M | 54.7 | 22.1 | 0.40 |
| | 12 | F | 47.3 | 13.8 | 0.29 |
| 120 | 8 | M | 45.6 | 17.7 | 0.39 |
| | 10 | F | 49.2 | 16.9 | 0.37 |
| 180 | 9 | M | 50.1 | 24.1 | 0.48 |
| | 8 | F | 53.8 | 21.0 | 0.39 |
| *C. Six days, beginning 4 hours after hatching* | | | | | |
| 0 | 15 | M | 44.6 | 12.7 | 0.29 |
| | 5 | F | 41.1 | 10.7 | 0.26 |
| 10 | 13 | M | 40.9 | 21.2 | 0.52 |
| | 6 | F | 44.2 | 19.0 | 0.43 |
| 20 | 8 | M | 47.8 | 24.5 | 0.51 |
| | 13 | F | 40.6 | 22.9 | 0.56 |
| 40 | 7 | M | 44.6 | 33.3 | 0.75 |
| | 12 | F | 45.9 | 30.7 | 0.62 |
| 60 | 7 | M | 49.4 | 44.0 | 0.89 |
| | 15 | F | 46.4 | 34.1 | 0.74 |
| 80 | 13 | M | 47.7 | 42.3 | 0.89 |
| | 12 | F | 46.9 | 43.0 | 0.92 |

ably, but smaller amounts introduce serious practical problems both of application and measurement.

White Leghorn chicks were used. The technic of Frank et al. was followed throughout the first group of experiments. In table 1 and figure 3 are the results from one of several experiments; these are typical of the series. The number of chicks used in any one experiment was not large because of cost, and also because of our hope of using this method in the assay of unknown samples. The amount of androgen obtainable from specimens of urine in clinical practice is usually too small to be assayed on a large number of test animals. The results show, as do Frank's and the more recent work of Danby (7), that the variation in response of the 7-day-old chick is too large to make them usuable in quantitative assay.

In order to see whether the use of 1-day-old chicks would improve the test, another series was run. Table 1 and figure 4 show the results. The response is no more dependable than in the foregoing experiments and led to the abandonment of the Frank technic.

In the next series of experiments the technic of Dorfman and Greulich was used, the androgen being injected subcutaneously. The variations in response that marred the former experiments appear here also as illustrated in table 2, and figure 5. If a comparison is made of the size of the male chicks' comb at 7 days with that of the female at the same time, it will be seen that Dorfman and Greulich are supported in their statement that the male chick has already started to grow its comb by that time. It would be better therefore to use only females or to start with younger chicks. With great trouble we obtained a large number of 4-hour-old chicks and injected them. The problem of getting a homogeneous group at that age is not a negligible one. The results are shown in table 2 and figure 6. Here again a wide variation in response is observed. This shows the chick to be an unreliable animal for androgen assay.

It should be noted that even in the last experiment, the females gave more consistent results than the males. This confirms our experience with roosters and 'slips' (incomplete capons) in which injections of androgens elicit highly variable response. It is difficult to ascribe this to the amount of testis tissue present. In these experiments, the male chicks showed a striking similarity to the 'slips.' Their response to injected androgen is not a simple one. The presence of their own androgen as well as the reaction of testicular tissue to the injection complicates the response. The female, without these complicating factors, responds more consistently to injected androgens, but not sufficiently to be of practical value.

## SUMMARY

The chick method for the assay of androgens has been tested for quantitative use. The variation in the response of the chick comb to known injections of androgens is so large in either the Frank et al. method or that of Dorfman and Greulich that quantitative androgen assay of known or unknown samples is impossible with this method.

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# THE USE OF THE CHICK FOR THE ASSAY OF ANDROGENS

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THE BIOASSAY of androgenic material can be accurately accomplished by the measurement of the size of the combs of capons following the administration of the hormone (1). Unfortunately, it is difficult and expensive to maintain a suitable flock of birds. As will be demonstrated in a subsequent publication, the chemical methods used in the quantitative estimation of ketosteroids do not always demonstrate the androgenic activity of a biologic mixture. For these reasons an attempt has been made to use the chick for the assay of androgens. Several reports (2–8) of similar work have already appeared in the literature. This paper includes the significant findings from this laboratory after the study of about 1,500 chicks.

Since our researches include the study of the amount of androgenic material in blood, we found it necessary to use the most sensitive method available. For this reason inunction was preferred to injection. In all the experiments reported here White Leghorn chicks were used.

Extensive preliminary experiments, which are not reported in detail, were carried out to determine the most satisfactory procedure to employ. The sensitivity of birds of different ages was tested, the quantity of oil which could be used was investigated and the number of days over which the assay should be run was studied. From the results of these experiments it was decided that birds 6 to 7 days of age should be used, because when older, the comb varies from bird to bird due to their own androgenic production, and when younger the mortality is fairly high. It also was shown that they should be inuncted daily for 5 days and that a longer test period did not increase the accuracy of the test. The dose could not be applied properly in more than 0.02 cc. of oil and it was preferable to use 0.01 cc. The oil was applied to the comb, using a microsyringe and a blunt needle. The syringe was held in a clamp and the operator manipulated the bird's comb about the needle as the oil was ejected. A micrometer screw was made of such a size and shape that a tuberculin syringe could be used. The syringes varied somewhat in caliber and therefore each syringe used was calibrated. When several assays were being performed simultaneously considerable time was saved if each batch of material being assayed was kept in a separate syringe. The same micrometer screw was used on each syringe.

We found, as did Frank (5), that it was necessary to isolate the control chicks which were not injected in order to prevent contamination of their combs with androgenic material from the inuncted birds. The birds were kept on wire netting in metal cages.

Large chicks were noted to give a slightly greater response to the hormone than small birds. For this reason the response to androsterone was expressed as a ratio of comb weight to body weight. The combs were carefully removed with a scalpel and the weight of the comb, expressed in milligrams, was divided by the weight of the

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TABLE 1. THE RESPONSE OF THE CHICK'S COMB TO INUNCTION OF ANDROSTERONE

| Group | Daily dose of androsterone | No. of birds | Response as ratio of comb to body weight |
|---|---|---|---|
| | γ | | |
| 1 | 0.0 | 112 | 0.35 |
| 2 | 0.5 | 73 | 0.45 |
| 3 | 1.0 | 98 | 0.56 |
| 4 | 1.5 | 60 | 0.57 |
| 5 | 2.0 | 100 | 0.76 |
| 6 | 4.0 | 86 | 0.90 |
| 7 | 8.0 | 64 | 1.05 |
| 8 | 16.0 | 34 | 1.01 |
| 9 | 30.0 | 36 | 1.12 |

chick, expressed in grams. This ratio of comb weight to body weight was found to vary with sex and averaged about 1.20 greater in male birds than in females. When possible it was found best to use only male chicks. However, when mixed groups were employed, the comb to body weight ratios of the females were multiplied by the above correction factor in order to make them comparable to the results from the male birds.

Table 1 and figure 1 give the dose response relationship obtaining using a total of 663 birds to which was administered doses of androsterone varying from 0 to 30γ daily. The response reached a maximum when the dose was 8γ of androsterone daily. The curve in the graph was calculated from the responses to the doses varying from 0 to 4γ daily, since it was only in this range that quantitative studies be made. The formula, $y=0.35+0.23x-0.02x^2$—in which y is the response (comb weight in milligrams divided by body weight in grams) and x the daily dose (expressed as gamma

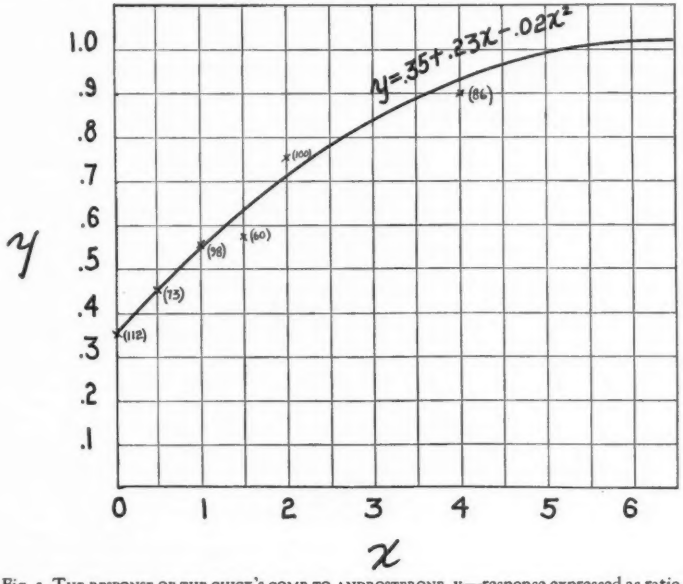


FIG. 1. THE RESPONSE OF THE CHICK'S COMB TO ANDROSTERONE. y—response expressed as ratio of comb to body weight. x—dose of androsterone in γ per bird in each group. The figures in brackets indicate the number of birds used to establish each point.

of androsterone) can be used in calculating the results of the assay of unknown preparations. If the greatest possible accuracy is to be obtained using this method for the assay of androgens, it is necessary to establish the exact nature of the dose response curve each time an assay or series of assays is made.

Unfortunately, it appears that there is such a large variation in response of chick's combs to the inunction of androsterone that this method cannot be relied upon for accurate quantitative investigations. For example, in table 1 it can be seen that even large groups of birds failed to show a significant difference in response to doses of 1.0

TABLE 2. ERRORS IN ASSAYS USING TEN CHICKS IN EACH GROUP

| Group | Daily dose of androsterone | Response as ratio of comb to body weight | Dose as calculated | Error |
|---|---|---|---|---|
| | γ | | | % |
| 1 | 4.0 | .94 | 3.86 | 3.5 |
| 2 | 1.0 | .83 | 2.75 | 175.0 |
| 3 | 4.0 | .90 | 3.51 | 12.2 |
| 4 | 2.0 | .72 | 1.94 | 3.0 |
| 5 | 1.5 | .78 | 2.35 | 56.6 |
| 6 | 1.0 | .62 | 1.32 | 32.0 |

and 1.5γ of androsterone daily. When small groups of birds are used the error sometimes is very high. Table 2 demonstrates the errors which occurred in 6 assays in which 10 chicks were employed for each assay. Three of the 6 assays were reasonably accurate. The errors in the other 3 were very large and indicate that the method is not dependable for quantitative measurements.

## SUMMARY

The assay of androgens by the method of inunction on the comb of the chick has been carefully studied and the most satisfactory technic established. Even when the greatest care is exercised it does not seem possible to obtain anything other than roughly quantitative results. The capon method continues to be the method of choice in the bioassay of androgens.

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# THE BABY COCKEREL TEST FOR ANDROGENS[1,2]

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THE INCREASE in weight of the comb of the White Leghorn chick during the first or second week of life as a result of the administration of androgens has been recommended by a number of workers as a reliable procedure for androgen assays (1–5). The methods of administrations used have varied from intramuscular or subcutaneous injections to direct application in oil solution on the comb. A

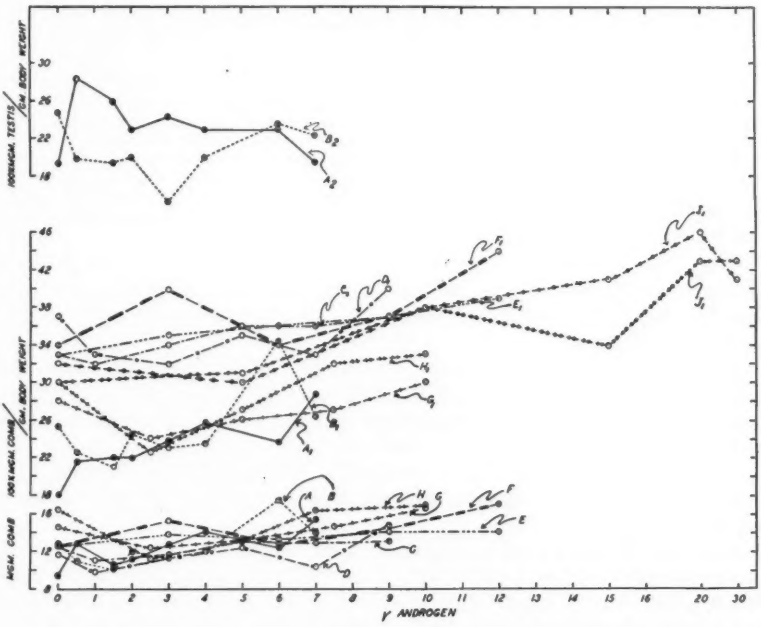


Fig. 1. THE COMB-WEIGHT RESPONSE IN BABY CHICKS TO THE INTRAMUSCULAR AND SUBCUTANEOUS INJECTION OF ANDROGENS. A, Average comb weights from ten 1-day-old chicks injected intramuscularly with 0.25 cc. olive oil solution of testosterone propionate daily, for 5 days. B, Duplicate of A. $A_1$, The quotient from mg. comb weight divided by grams body weight, not eviscerated, for the A group. $B_1$, The

(*Legend continued on next page*)

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[2] We are grateful to Dr. Erwin Schwenk of the Schering Corporation for the androgens used in this study.

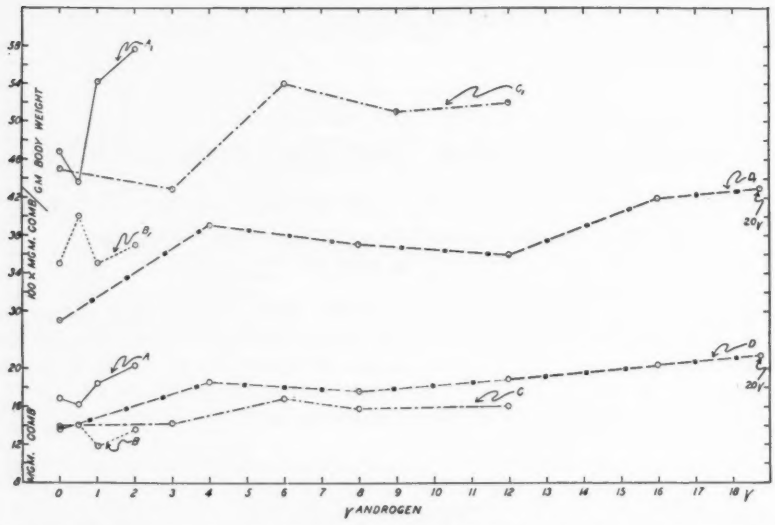


Fig. 2. THE COMB-WEIGHT RESPONSE IN THE BABY CHICKS TO THE LOCAL APPLICATION OF ANDROGENS. A, Average comb weights from twenty 1-day-old chicks injected into comb with 0.05 cc. daily for 5 days with an olive oil solution of testosterone propionate. $A_1$, The quotient from mg. comb weight divided by grams body weight (eviscerated) from group A. B, Same as A above, but the oil solution was applied on the comb. $B_1$, Same as $A_1$, but for group B. C, Average comb weights from 20 1-day-old chicks injected into comb with 0.05 cc. daily for three days with olive oil solution of testosterone. $C_1$, Same as $B_1$, but for group C. D, Average comb weights from 20 1-day-old chicks injected into comb with 0.02 cc. daily for 4 days with a propylene glycol solution of androsterone. $D_1$, Same quotient as in $C_1$, but for group D.

priori one would expect that the method must involve two uncontrollable variables. These are the original size of the comb and the variable comb growth produced by the chick's own testes.

Numerous attempts by us to prove the reliability of the method have convinced us that it is not as reliable as the original capon method. The results of our assays are presented in figures 1 and 2. The legends give the details as to the specific androgen, the mode of administration, etc. The chicks were single comb White Leghorns from the same hatchery. They were kept in brooders provided with false bottoms. The combs were weighed on a sensitive torsion balance immediately after careful dissection with small scissors.

Inspection of figures 1 and 2 shows that *a*), the necessary base lines, the controls, vary considerably even in duplicate groups observed at the same time; *b*), the comb growth responses from intramuscular or subcutaneous injections are of a lower order than from the treatment of the comb by direct injection therein or local application

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same for the B group. $A_2$, The quotient from mg. testis weight divided by grams body weight for the A group. $B_2$, The same for the B group. C, Same as A, but volume of olive oil solution reduced to 0.05 cc. per day for 5 days. D, Duplicate of C. $C_1$, The quotient from mg. comb divided by grams body weight (eviscerated) for the C group. $D_1$, Duplicate of $C_1$. E, Same as C, but used testosterone in place of the propionate and sesame oil in place of olive oil. F, Duplicate of E. $E_1$, The quotient from mg. comb divided by grams body weight (eviscerated) for the E group. $F_1$, Duplicate of $E_1$. G, Average comb weights on ten 1-day-old chicks injected subcutaneously through pectoral muscle on alternate sides for 5 days with 0.2 cc. daily of olive oil solution of androsterone. H, Duplicate of G. $G_1$, The quotient from mg. comb divided by grams body weight (eviscerated) for the G group. $H_1$, Duplicate of $G_1$. $I_1$, Same as $G_1$ and $H_1$, but injected for 6 days and sacrificed on the seventh day. Averages on 20 chicks. $J_1$, Duplicate of $I_1$.

thereon of the androgen solution; and *c*), the comb growth responses are very irregular by both methods of treatment. It is obvious that the controls are of dubious value although very necessary. The graphs in figure 2 suggest that the comb can grow only up to a certain rate and that higher levels of androgen applied locally do not increase the rate of comb growth to an appreciable degree. Unpublished data from this laboratory fully confirm this observation. The marked variations in the comb growth responses given in figures 1 and 2 may easily be due in part to the chick's own testicular secretion. This is suggested by comparison of $A_1$ and $B_1$ with $A_2$ and $B_2$ in figure 1.

We conclude that in our hands and with the strain of baby chicks used by us the baby chick androgen method is of doubtful value.

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# INFLUENCE OF TESTOSTERONE ON DISTRIBUTION AND EXCRETION OF CREATINE

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THE RELATIONSHIP existing between the testicular hormone and creatine metabolism, or more especially creatinuria, has been studied by a number of investigators (1–5). Most recently the papers by Beard and collaborators (6, 7, 8) have sought to elucidate the mechanism of this relationship. They consider that testosterone will cause a partial reconversion of creatinine into creatine in vivo, and that injected creatine is not directly converted to creatinine.

Thus far the work in this field has been concerned chiefly with the effects produced by testicular hormones or extracts on the urinary level of creatinine and creatine. Although these results tell a valid story, we believe that a more fundamental picture of this relationship can be obtained by considering at the same time with the urine the blood and muscular levels of creatine and creatinine.

## EXPERIMENTAL

Five series of experiments were performed on male rabbits. Throughout the experiments they were kept on a comparatively high protein diet in order to reinforce

TABLE 1. EXPERIMENTAL SERIES 3, NO CREATINE

| Before hormone injections | | | After hormone injections | | | | |
|---|---|---|---|---|---|---|---|
| Muscle | Total creatinine | % | Muscle | Total creatinine | % | Increase | Relative increase |
| gm. | mg. | | gm. | mg. | | | % |
| 0.794 | 2.70 | 0.34 | 0.735 | 2.88 | 0.39 | 0.05 | 15 |
| 1.457 | 3.49 | 0.24 | 1.494 | 4.36 | 0.29 | 0.05 | 21 |
| 0.996 | 2.91 | 0.29 | 1.031 | 3.25 | 0.31 | 0.02 | 7 |

TABLE 2. EXPERIMENTAL SERIES 4, 1500 MG. CREATINE

| Before injections | | | After injecting hormone+1500 mg. creatine | | | | |
|---|---|---|---|---|---|---|---|
| Muscle | Total creatinine | % | Muscle | Total creatinine | % | Increase | Relative increase |
| gm. | mg. | | gm. | mg. | | | % |
| 1.014 | 2.48 | 0.24 | 1.060 | 3.96 | 0.37 | 0.13 | 54 |
| 0.700 | 2.29 | 0.33 | 0.671 | 2.86 | 0.42 | 0.09 | 27 |
| 0.722 | 2.11 | 0.29 | 0.705 | 2.97 | 0.42 | 0.13 | 45 |

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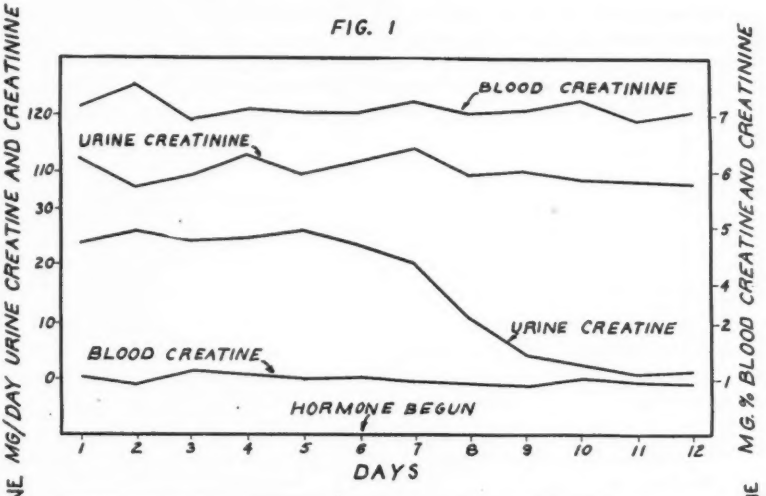
any creatinuria which may have been normally present. During experimental periods they received oreton (testosterone propionate) by daily subcutaneous injections at the rate of 2.5 mg. of the compound per day.

Creatinine and creatine were determined according to the micro-method of Folin

FIG. 1



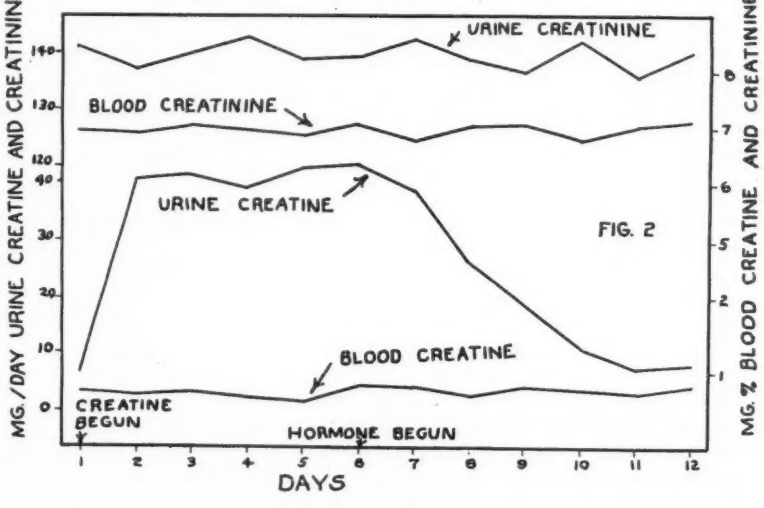



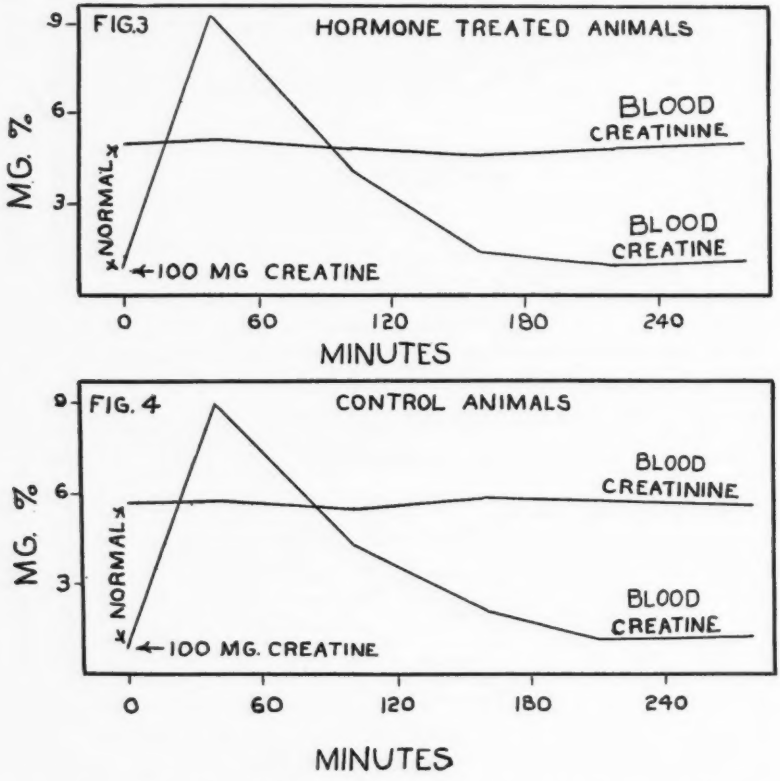

FIG. 2

(9, 10) in the blood and urine samples of experimental series 1, 2, 5. The total muscle creatine-creatinine in experimental series 3 and 4 were determined by the method of Rose, Helmer and Chanutin (11).

In series 1 (fig. 1), the urine was collected in 24-hour samples, free from food and feces. A blood sample was taken from an ear vein at the end of each 24-hour period. After a number of days, oreton was injected daily until a definite effect was noted.

In series 2 (fig. 2), this same procedure was followed with the exception that approximately 50 mg. of creatine in isotonic saline was injected daily immediately after the blood and urine samples were obtained.

In series 3 (table 1), the flexor carpi ulnaris was removed by sterile procedure from one foreleg. After 12 hours, an injection of hormone was given. The hormone dose was repeated every 24 hours until the end of the experiment. Four days later, the same muscle on the opposite leg was removed. In series 4 (table 2), this same procedure




was used except that in addition a total of 1500 mg. of creatine in 500 mg. doses was injected during the course of the 4-day period.

In series 5 (fig. 3), the rabbits were given the hormone approximately 24 and 4 hours before the experiment. Then 100 mg. of creatine was injected intravenously, and blood samples were taken at hourly intervals. Control rabbits (fig. 4) were treated in the same manner except that they did not receive any hormone.

## RESULTS AND DISCUSSION

An examination of figures 1 and 2 (representing series 1 and 2) shows one outstanding feature. Regardless of the type of treatment, the blood creatine and creatinine remained strikingly constant, within experimental limits. Since creatinine is recognized to be a non-threshold substance, which is therefore gradually and con-

tinually stripped from the blood, it must be concluded from the constancy of the circulating and urinary creatinine that there must be a continuous supply of creatinine flowing into the blood as a result of tissue metabolism. On the other hand, the constancy of the blood creatine can be explained only if, in contrast to creatinine, it is considered to be a threshold substance, since it remains at one level in the blood in spite of the hormone treatment which nearly or quite put an end to the excretion of creatine in the urine. Series 5 (fig. 3, 4) bears this out further. Without considering the destination, the extra creatine is removed from the blood within approximately 3 hours and only the original level remains. It is to be noted that at the hormone level used, there is no difference in reaction in this experiment between the treated and the control animals.

Within the limits of the errors that occur in the collecting of 24-hour urine samples, the urinary creatinine was found to be fairly uniform from day to day. This is contrary to what is to be expected in view of the work of Pizzolato and Beard (6) who infer a reconversion of creatinine into creatine. It is in agreement, however, with the later brief paper (7) by Beard and Jacob.

When creatine was injected, part of it was excreted in the urine and part of it was retained. But, within 24 hours after injection, all excess creatine had disappeared from the blood. Evidently that portion which had not been eliminated by excretion had been disposed of in some other manner, most likely by absorption into some tissue or organ. When the testosterone was administered, this non-excretory disposal of creatine was enhanced—to the point where almost none of the creatine was excreted, regardless of its origin. This effect is noticeable in the experiments of series 1, where no extra creatine was given, as well as series 2, although not to so marked a degree.

Since part of the injected creatine remains unexcreted, especially after testosterone administration, the most likely place for its deposition is in the muscles. It can be safely said that if there is any rise in creatine content of the muscle of the experimental animals, it is due to the effect of the hormone, since feeding of creatine is found to have very little effect on its concentration in the muscle tissue (12, 13). In series 4 (table 2) where creatine was given as well as testosterone, there was a greater increase in muscle creatine than was found in series 3 (table 1) where no creatine was injected. Before this creatine could reach the muscle for deposition or the kidney for excretion, it must perforce have traveled through the blood. This seems to point to the fact that creatine is actively recalled from the blood by the action of the hormone.

Since creatine is continually being synthesized to make up for that which is being lost in the form of creatinine (14, 15), it is not too difficult to understand the increases to be found in series 3. Besides recalling the creatine from the blood, the testosterone seems to make the muscle better able to hold the creatine or possibly increases the capacity of the muscle for creatine. This may well be a part of the mechanism for the improvement in dynamometric response which Hesser, Langworthy and Vest (16) report they find with the administration of testosterone in cases of myotonia atrophica.

## SUMMARY

When male rabbits are given testosterone, with or without the administration of creatine, the testosterone diverts creatine into the muscle, or causes its cumulative retention in the muscles. Thus it lowers urinary creatine and raises muscle creatine, but without appreciably changing the creatine content in the blood.

In contrast to creatinine, creatine behaves as a threshold substance, although having a low threshold.

The creatine content of the blood is held very uniform, and even when it is

drastically altered by the intravenous injection of creatine, the figure rapidly returns to normal.

Within the time and dosage limits of these experiments, the creatinine metabolism is not detectably influenced by testosterone, nor by the abnormal fluctuations of blood and muscle creatine which our experiments produced.

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# THE EXPERIMENTAL PRODUCTION OF GLANDULAR CYSTIC HYPERPLASIA IN CASTRATED MACAQUES[1]

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GLANDULAR CYSTIC HYPERPLASIA of the endometrium has been produced experimentally by the action of estrogenic substances in women (1–4) and in some of the laboratory animals (5–10). However, there has been a lack of uniformity in the reports of investigators, some of whom have not observed the condition in monkeys treated with large doses of estrogen, for long periods of time. As a result of these different reports (10–15) a certain degree of confusion concerning the etiology of glandular cystic hyperplasia has arisen. The amounts of estrogenic material and the length of the periods of treatment reported in these experiments were varied, so that a table of the available data was compiled in order to simplify the survey (table 1). The differences in the resulting observations may, in some measure, be due to differences in the interpretation of the histology of the specimens of tissue from the experimental animals.

The histologic characteristics of glandular cystic hyperplasia were enumerated by Fluhman (16) in a paper appearing in 1935. Briefly, these characteristics are: *a*) an appearance of the tissue suggesting an intense growth activity; *b*) a uniformity in the appearance of the tissue which makes for difficulty in differentiating the basal and functional regions of the endometrium; *c*) an extremely cellular stroma composed of spindle shaped cells; *d*) the occurrence of large blood vessels throughout the endometrium in contrast to their confinement in the deeper areas of normally proliferating endometrium; *e*) the presence of numerous mitotic figures in the stroma; *f*) an increased number of glands whose size and shape in cross section varies, and which are arranged in a disorderly manner; g) gland epithelium which may be of the simple or pseudostratified type, and may be low cuboidal in form in the various cystic glands.

## PROCEDURE

Six castrated female macaques were employed in this experiment to determine whether or not glandular cystic hyperplasia of the endometrium could be produced by the unopposed action of large amounts of estrogenic material. Dosage levels of the hormone,[2] were chosen which were far in excess of those reported in the literature, i.e. 2,500 and 5,000 R.U. estrogen each day. Three months was chosen as an experimental period of time which was approximately intermediate between that of the shorter and longer periods of treatment reported in other experiments. At the close of the ex-

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[2] The estrogen in the form of progynon-B was made available through the courtesy of Dr. Erwin Schwenk of the Schering Corporation.

periments biopsies of the endometrium were secured by laparotomy. The pertinent data are incorporated in table 2.

The estrogen was administered subcutaneously daily, with the exception of Sunday. The injections, made into the hind leg of the animals, produced no local irritation. All of the animals showed a gain in weight for the first few weeks of the experiments, after which time some of them lost as much as 0.5 kg. The swelling of the sexual skin varied in degree in the different animals, but the excessive swelling subsided within a few weeks, giving way to low fibrous corrugations of the remaining

TABLE 1. REPORTS IN LITERATURE ON PRODUCTION OF GLANDULAR CYSTIC HYPERPLASIA IN MONKEYS

| Investigators | Animal No. | Wt., kg. | Previously biopsied | Material used | Type units | Dosage | | Period of treatment, days | Results | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Daily | Total | | Glands | Stroma |
| Tietze, K. '34 | 4 | 4.5 | ? | Menformon in oil: intramuscular | Mouse units | — | 72,000 | 72 | Glandular cystic hyperplasia | |
| Engle and Smith '35 | M 169 | 5.0 | No | Squibb's amniotin in oil | R.U. | — | 23,400 | 60 | — | — |
| | M 146 | 3.6 | No | | R.U. | — | 25,900 | 80 | — | — |
| | M 170 | 4.4 | No | | R.U. | — | 37,400 | 110 | — | — |
| | M 165 | 4.6 | No[1] | | R.U. | — | 95,400 | 170 | Dilated | — |
| Hisaw '35 | M 6 | ? | ? | Estrin, undesignated | R.U. | — | 1,220 | 31 | — | — |
| | Al 28 | ? | ? | | R.U. | — | 8,240 | 103 | — | — |
| Zuckerman and Morse '35 | Chimp | ? | No | Estrin from Schering and Parke, Davis | R.U. | — | 21,100 | 20 | ++ | Dense |
| | Mangabey | ? | No | | R.U. | — | 33,675 | 40 | + | Dense |
| Zuckermen '37 | O M 98 | 3.4 | No | Crystalline estrone in oil, Schering | γ | — | 36,500 | 365 | + | + |
| Engle and Smith '38 | My 209 | 5.4 | No | Progynon-B | R.U. | 1000 | 135,000 | 135 | "Prophetic" | |
| | My 271 | 5.1 | No | Crystalline estrone in oil, Schering | R.U. | — | 76,500 | 54 | — | — |
| | My 208 | 7.3 | No | | R.U. | — | 135,000 | 82 | — | — |
| | My 286 | ? | ? | | R.U. | — | 31,000 | 30 | — | — |

[1] Biopsied on 67th day of experiment.

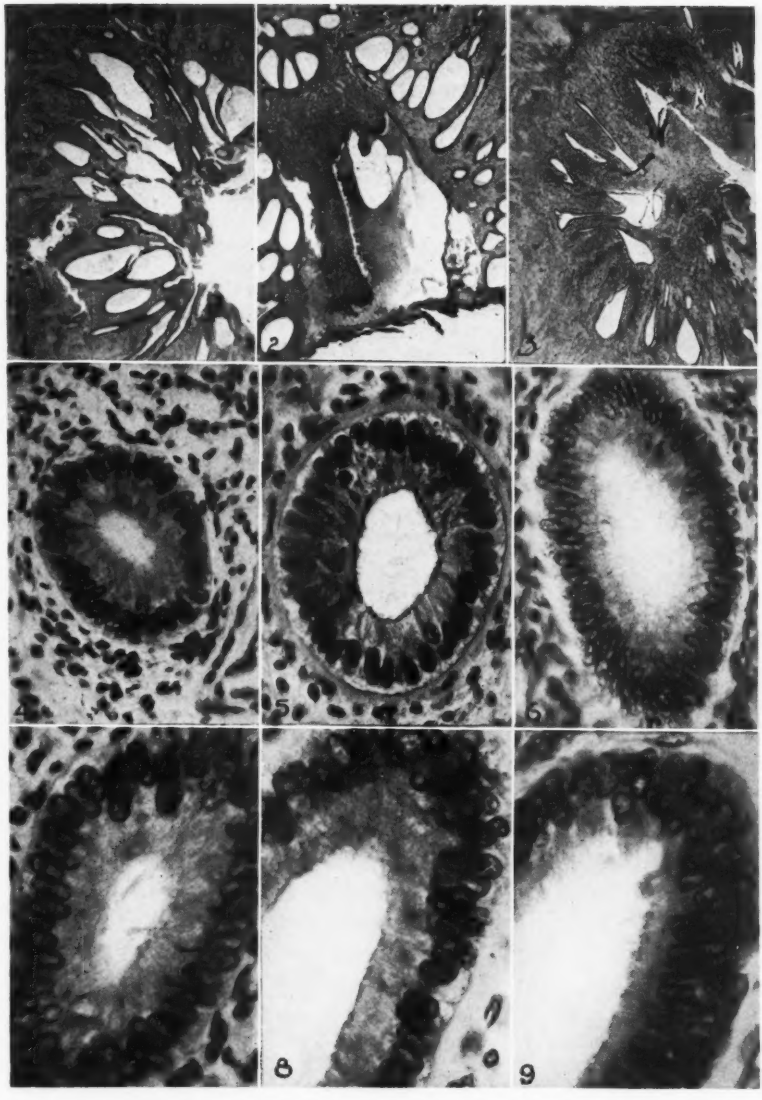
skin of the body, extending over the entire back, the shoulders, head and face of the animals.

The biopsies of the endometrium were removed by laparotomy 24 hours after the last injection of estrogen. Measurements were made at the longest and the widest points of the body of the uterus. No significance could be given to the measurements of length and width of the uteri at this time since 3 of the animals had been biopsied 4 and 6 times in previous experiments and only small remnants of the uterine tissue were present in these animals at the outset of this experiment.

The tissues were promptly fixed in Bouin's fluid and prepared for histologic study by the routine procedure employed in the laboratory of the Department of Obstetrics and Gynecology.

## RESULTS

All six of the specimens of endometrium obtained at the conclusion of the period of treatment with estrogen exhibited the histologic characteristics of proliferating




ALL PHOTOGRAPHS ARE OF ENDOMETRIUM FROM CASTRATED MACAQUES TREATED WITH ESTROGEN FOR 3 MONTHS. Tissues fixed in Bouin's fluid and stained with hematoxylin and eosin. Wratten plates and K2 filter. Fig. 1–3. LOW POWER VIEWS OF SECTIONS OF ENDOMETRIUM FROM ANIMALS HAVING GLANDULAR CYSTIC HYPERPLASIA. Magnification ×25. Fig. 1. *M.*10. 2,500 R.U. estrogen daily for 3 months; 4 previous biopsies. Fig. 2. *M.*9, 5,000 R.U. estrogen daily for 3 months; 6 previous biopsies. Fig. 3. *M.*4, 5,000 R.U. estrogen daily for 3 months; 6 previous biopsies.

Fig. 4–9. SMALL GLANDS FROM THE ENDOMETRIUM OF MACAQUES HAVING GLANDULAR CYSTIC HYPERPLASIA. Note the characteristics of proliferation in the epithelium. Fig. 4, 5, 6, ×340. Fig. 7, 8, 9, ×560. Fig. 4, 7. Glands from *M.*10, 2,500 R.U. estrogen daily. Fig. 5, 8. Glands from *M.*9, 5,000 R.U. estrogen daily. Fig. 6, 9. Glands from *M.*4, 5,000 R.U. estrogen daily.

endometrium. However, only 3 of these 6 tissues exhibited the excessive degree of proliferation associated with glandular cystic hyperplasia. For the purposes of description the experimental animals have been divided into groups *A* and *B*, on the basis of the dosage levels of estrogen (2,500 R.U. per day, and 5,000 R.U. per day).

*Group A.* The 3 animals in this group received 2,500 R.U. estrogen daily for approximately three months (table 2). Glandular cystic hyperplasia occurred in the endometrium of but one of these macaques, *M. 10.* This adolescent animal had been castrated and biopsies had been secured by laparotomy 4 times prior to use in this particular experiment. The remaining 2 animals, in which there was no evidence of cystic

TABLE 2. EXPERIMENTAL DATA ON PRODUCTION OF GLANDULAR CYSTIC HYPERPLASIA IN CASTRATED MACAQUES

| Group | Animal No. | Hyperplasia produced | Approximate age at time of castration | No. of times previously biopsied[1] | Weeks elapsing since last biopsy | Wt. at outset of exp. | Material injected | Daily dosage | Total dosage | Total period of exp. | Uterine measurement at end of exp. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *kg.* | | R.U. | R.U. | *days* | *cm.* |
| *A* | *M 10* | + | Adolescent | 4 | 4 | 5.2 | Progynon-B (in oil) Schering Corp. | 2,500 | 212,500 | 99 | — |
| | *M 15* | − | Preadolescent | 0 | — | 4.2 | | | 192,500 | 91 | 2.5×2 |
| | *M 16* | − | Adolescent | 0 | — | 5.1 | | | 195,000 | 92 | 2×1.5 |
| *B* | *M 4* | + | Mature | 6 | 4 | 3.9 | Progynon-B (in oil) Schering Corp. | 5,000 | 425,000 | 98 | — |
| | *M 9* | + | Preadolescent | 6 | 12 | 3.5 | | | 385,000 | 91 | 2×2 |
| | *M 14* | − | Adolescent | 1 | 12 | 4.2 | | | 390,000 | 92 | 3×2 |

[1] All biopsies secured by laparotomy.

dilatation of the glands, had been castrated a few weeks before this experiment was begun, and no biopsies of the endometrium had been attempted.

*Group B.* A daily injection of 5,000 R.U. estrogen was given each of the 3 animals in this group for a period of time approximating 3 months (table 2). Glandular cystic hyperplasia occurred in 2 of the 3 members of this group, *M. 4* and *M. 9.* Both of these animals had been employed in previous studies, and each had been biopsied by laparotomy 6 times before this experiment was undertaken. The third animal, *M. 14,* in which there was no glandular cystic hyperplasia, had been castrated and biopsied once prior to use in this experiment.

## DISCUSSION

That glandular cystic hyperplasia was produced in 3 of 6 castrated macaques by the use of what was considered massive doses of estrogen, further supports the concept that the etiology of glandular cystic hyperplasia lies in the unopposed action of estrogenic substances on the endometrium.

When the experimental data were classified on the basis of the number of biopsies secured from the animals prior to the initiation of this experiment, it became apparent that hyperplasia resulted in those animals from which substantial amounts of basal and functional endometrium had been removed in previous experiments. Thus it appears that the ratio of available estrogen to the amount of existent endometrium may be a factor in the experimental production of glandular cystic hyperplasia.

Engle and Smith (11), in an experiment in which the level of estrogen was periodically increased over a period of 170 days, removed a biopsy of the endometrium from a macaque on day 67 of the experiment. At the conclusion of the experiment, a second

biopsy of the endometrium revealed that the tissue had the characteristics of glandular cystic hyperplasia, but the authors hesitated to accept the result as 'positive' on the basis that the surgical interference may have played a role in producing the dilated glands. It is possible that the increasing doses of estrogen, combined with the surgical reduction of the uterine tissue, brought about a balance between the amount of estrogen and of endometrial tissue which was adequate for producing the dilated glands characteristic of endometrial hyperplasia.

Although the age and general constitutional state of the animals employed in the current study may have been responsible for the success and failure to produce hyperplasia experimentally, the series of animals was too small to reveal the relationship.

## CONCLUSIONS

Glandular cystic hyperplasia may be produced experimentally in the endometrium of castrated macaques by the action of estrogenic substances.

The ratio of estrogenic material to the amount of the endometrial tissue may be an important factor in producing the condition. But whether the proportion of estrogen to the volume of endometrium is one of an excess of estrogen in the presence of a relatively normal amount of endometrial tissue, or whether the proportion is one of an excess of estrogen to a volume of endometrium far less in amount than is characteristic for the normal macaque, is a subject for further investigation.

## SUMMARY

Glandular cystic hyperplasia was produced in three of six castrated macaques treated with massive daily doses of estrogen (2,500 and 5,000 R.U. per day) for a period of 3 months. The cystic dilatation of the glands occurred in animals which had been subjected to biopsy by laparotomy 4 and 6 times prior to the initiation of the current experiment. Animals which had been biopsied once or not at all did not have glandular cystic hyperplasia of the endometrium at the conclusion of the experiment.

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# CONTINUOUS AND DISCONTINUOUS TREATMENT WITH ESTROGENS IN EXPERIMENTAL TUMORIGENESIS[1]

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NELSON (1, 2) and later on Moricard and Cauchoix (3) stated that uterine fibroids can be produced in the guinea pig by prolonged treatment with estrogens. Lipschütz and Iglesias (4, 5) established that under certain quantitative and timing conditions fibroids originate in most of the organs of the abdominal cavity of guinea pigs treated with estrogens. As reported by Vargas and Lipschütz (6) these experimental abdominal fibroids can be found in the abdominal cavity even in absence of uterine tumors. All of the available estrogens, natural, esterified and artificial, are tumorigenic, but there is a remarkable difference between free and esterified hormones (7, 8, 9). Free hormones are less tumorigenic than the esterified ones when equal quantities of the hormones are compared. The difference between a 'physiological' hysterotrophic dosage and the tumorigenic one is also smaller with the esterified hormone than with the free one. To explain these differences Lipschütz (10) suggested that a stable folliculinemia is probably more easily established by the esterified hormone. The free hormone is rapidly absorbed from the site of injection but is also rapidly inactivated and eliminated; on the contrary, the absorption of the esterified hormone is slow and when injecting it every second day, as in our experiments, a continuous flow of the hormone into the blood will probably be established. Proof in favor of these asumptions was given by Lipschütz and Vargas (11) with tablets of estradiol introduced beneath the skin by the technic of Deanesly and Parkes (12); under these experimental conditions the flow from the tablet into the blood is a continuous one and the free hormone showed itself to be more tumorigenic than in experiments with subcutaneous injections of the same hormone repeated every second day. Further proof was given by Lispchütz and Vargas (13) in experiments with stilbestrol which as shown by these authors has a greater tumorigenic action than free estradiol; according to Zondek and Sulman (14) absorption of stilboestrol from the site of injection is slow and as shown by Stroud (15) stilbestrol is more resistant against inactivation in the body than the natural estrogens.

If the greater tumorigenic action of the injected esterified hormone as compared with that of the free one is due to the greater facility with which a continuous flow is established in the body with the former, it should be possible to deprive the esterified hormone of its tumorigenic faculty in experiments with a prolonged treatment, by allowing for intervals free of injections, i.e., by allowing for a diminution of the folliculinemia in these intervals. It was also thought that similar experiments might be of a considerable interest from a clinical point of view.

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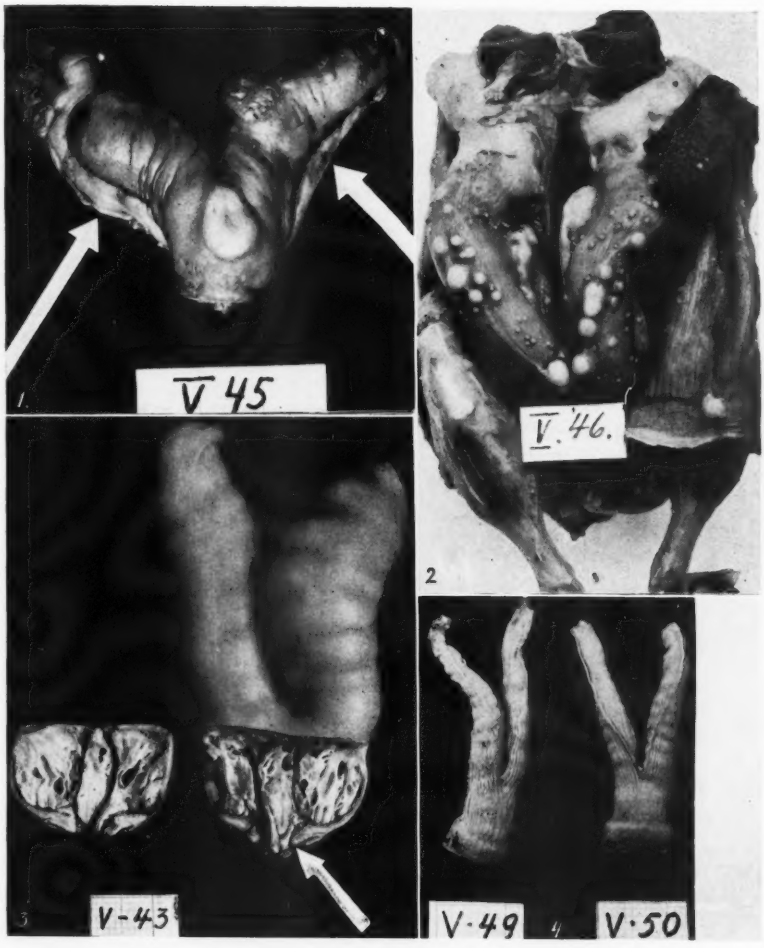


Fig. 1. Uterus of castrated guinea pig receiving 20γ of estradiol monobenzoate thrice weekly in the course of 16 weeks (47 injections; total 940γ). Enormous development of uterus. Big saddle tumor on the uterine base and between uterine horns. Enormous parametric tumors (arrows) covering also dorsal face of the horns. Tumors on the left horn (with adherent epiploon). Tubal cyst of the right horn.

Fig. 2. Uterus of castrated guinea pig receiving 20γ of estradiol monobenzoate thrice weekly in the course of 16 weeks (47 injections; total 940γ). Great number of subserous tumors on both horns; enormous parametric and 'apical' tumors reaching the kidneys. Subserous tumor of abdominal wall on the left (arrow). See also spleen with 'tumoral seed' on the surface.

Fig. 3. Uterus of castrated guinea pig receiving 20γ of estradiol monobenzoate thrice weekly in the course of 45 weeks (116 injections; total 2320γ). Enormous development of uterus (26 gm., instead of 1.0 to 1.5 gm. in the normal virginal animal). Septum between horns greatly thickened; cystic uterine glands and polyps extending into the vagina. See also fig. 8-A and 8-B.

Fig. 4. Uteri of castrated guinea pigs receiving 80γ of estradiol monobenzoate thrice weekly in the course of 365 days. Each week of injections (16 weeks) was followed by 15 to 19 days without treatment (47 injections; total 3760γ). Uterine wt. 1.4 and 1.7 gm. Sacrificed 20 days after last injection. Normal aspect of uterus. See also fig. 7.

*Administration of Estradiol for 113 to 312 Days without Intervals*

Six castrated female guinea pigs (350 to 615 gm.) received thrice weekly subcutaneous injections of 20γ of esterified estradiol monobenzoate. Three animals of this group having developed palpable uterine tumors were killed 113 days after the beginning of the treatment during which 37 injections of a total of 940 of estradiol were given. Enormous uterine and extrauterine tumors were found at the postmortem examination (fig. 1, 2B). The total tumoral index was high, between 6 and 12, according to our system of marks. Uterine development was very considerable; the weight of the uterus without tumors was in one case 7.4 gm., and in another case 23 gm. (including uterine tumors) (fig. 1). The other 3 animals of this group did not show palpable tumors and the experiment was prolonged in one case for 155 days (63 injections, total quantity of estradiol injected—1260γ) and for 312 days in the remaining two cases (116 injections, total quantity injected, 2320γ). A big apical tumor was found at postmortem examination in one case and small subserous uterine fibroids in 2 cases. Uterine development became enormous and even monstrous (fig. 3). In the latter case the uterus which was without tumors had a weight of 26 gm. A seventh animal received 80γ of estradiol monobenzoate thrice weekly and was killed 79 days after beginning of the treatment (34 injections; total, 2720γ of estradiol). There were uterine and extrauterine abdominal tumors.

The atypical growth of the uterine mucosa in all these experiments deserves special mention. The uterine was filled with polyps (fig. 5-A) or enormous cystic glands (fig. 8-A). Sometimes the hypertrophic mucosa revealed an adenomatous aspect (fig. 5-B). These uterine changes appeared also in the animal treated for 79 days only. In several cases the uterine polyps extended into the vagina (fig. 1, 3, 8-B).

In 6 of the 7 animals uterine bleeding appeared repeatedly 2 to 4 months after beginning of injections.

*Administration of Estradiol for 365 Days with Intervals*

Six castrated female guinea pigs (350 to 660 gm.) received thrice weekly subcutaneous injections of 20γ of esterified estradiol monobenzoate, as in the above group, but with the difference that every week of treatment was followed by an interval free of injections. The interval was prolonged till the vagina which opened after the first injection again closed; an interval of 15 to 19 days was sufficient for this. In this way each animal received 47 injections of 20γ or a total of 940γ in the course of 365 days; there were 16 weeks of injections separated from one another by a total of 36 weeks free of injections. Two other castrated guinea pigs (370 and 390 gm.) were injected thrice weekly with 80γ of estradiol monobenzoate, each week of injections being followed by 15 to 19 days free of injections; there were as in the former group 16 weeks of injections (47 injections, total 3760γ) and 36 weeks free of injections.

None of the 8 animals had uterine bleeding and there were no palpable tumors. They were killed 365 days after beginning of treatment, 20 days after the last injection. In no animal uterine or extrauterine fibroids were found at post mortem examination. The uterus in all the animals revealed a normal macroscopical aspect (fig. 4). The average uterine weight was 1.6 gm. (1.2 to 1.4 gm. in 4 cases, and 1.7 to 2.0 gm. in the remaining 4 cases).

Microscopical examination was made in all the 8 animals of this group. Pluristratification of the vaginal mucosa was still present in 7 although 20 days had elapsed since the last injection; in one case there was still cornification. These findings show that the interval of 15 to 19 days was not sufficient to allow for inactivation and elimination of all the estrogen administered during the week of injections but only for a considerable diminution. In several instances there was also great development of the epithelium of the portio with ingrowth of epithelial cords into the submucosa; in 2 cases these cords offered a precancerous aspect (fig. 9). Glandular cysts were

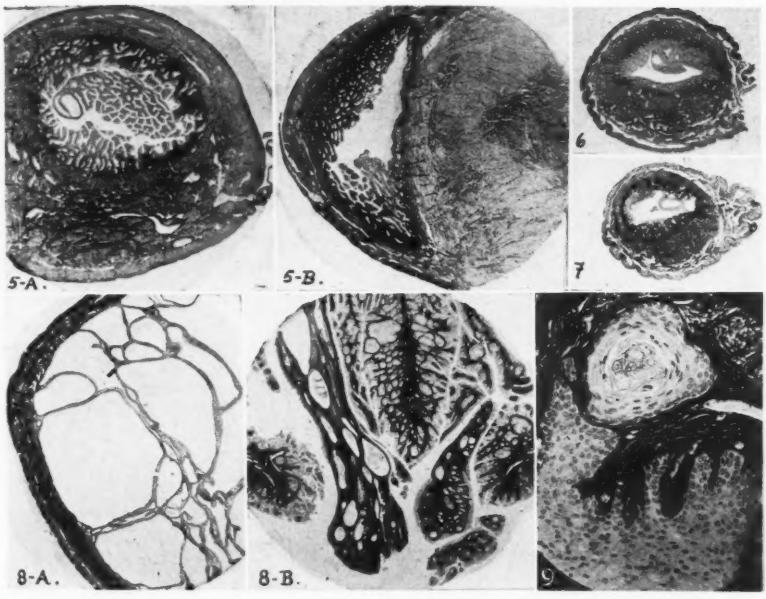


Fig. 5. Uteri of castrated guinea pig receiving 20γ of estradiol monobenzoate thrice weekly in the course of 16 weeks. *A*. Great development of myometrium. Polyps filling uterine cavity. *B*. Adenomatous development of endometrium. Uterus compressed by an enormous parametric tumor.

Fig. 6. Uterus of castrated guinea pig receiving 20γ of estradiol monobenzoate thrice weekly in the course of 365 days. Each week of injections (16 weeks) was followed by 15 to 19 days without treatment. Sacrificed twenty one days after last injection. Slight glandular hyperplasia. To be compared with fig. 6, 8; same magnification.

Fig. 7. Same animal as in fig. 4. (V, 49) Eighty γ per injection, 47 injections in the course of 365 days. Slight glandular hyperplasia. To be compared with fig. 6, 8; same magnification.

Fig. 8. Same animal as fig. 3. *A*. Transverse section of uterine horn. Enormous glandular cysts. *B*. Frontal section of uterine base. Uterine polyps extending into the vagina.

Fig. 9. Castrated guinea pig receiving 20γ of estradiol monobenzoate thrice weekly in the course of 365 days. Each week of injections was followed by 15 to 19 days free of injections. Sacrificed twenty days after last injection. Wt. of uterus 1.8 gm. Vagina closed. Fornix with pluristratified epithelium and ingrowth of epithelial cord. Concentric disposition of cells in the cord; vacuolated cells in the midth ofthe cord.

Fig. 5 to 8, 4.4×; fig. 9, 88×.

present in the transitional zone between the individual cervix and the uterine horn in most animals of this group. But the endometrium of the uterine horn itself was mostly normal and only in some cases there was slight hyperplasia with cysts (fig. 6, 7). The mammary glands in most of the animals were in a state of proliferation, characteristic of the beginning of pregnancy in the guinea pig.

## DISCUSSION

Our experiments give full evidence that the same quantity of esterified estradiol which produces uterine and extrauterine fibroids, monstrous development of the uterus and uterine bleeding when continuously injected in the course of 3 to 4 months, loses the faculty to produce these toxic uterine and extrauterine effects if injected discontinuously, i.e., if every week of injections is followed by a convenient interval free of hormonic treatment. In experiments of Bellolio (16) fibroids were induced by a continuous injection of quantities so small as 5γ per injection or a total of 200γ in a course of 3 months; uterine polyps may appear already with 1γ per injection or a total of 40γ (17). On the contrary, in those experiments of the present investigation in which injection was discontinuous, quantities many times greater

(a total of 940 to 3760γ in the course of 1 year) appeared to be still unable to induce fibroids or a monstrous development of the uterine mucosa. It becomes clear that toxic actions of estrogens are established when these substances are administered in such a way that the normal sequence of follicular and non-follicular phases as characteristic for the normal sexual cycle is experimentally overthrown. A replacement therapy with estrogens must duly allow for periods free of treatment so as to imitate the rhythm of normal endocrine ovarian function (18, 19).

There is still another interesting feature in these experiments. Periods of 15 to 19 days free of injections were, in our experimental conditions, sufficient to avoid production of uterine and extrauterine fibroids, the monstrous development of the uterus and uterine bleeding; nevertheless the uterine mucosa was not always normal. There was an atypical proliferation of the epithelium in the portio and in the transitional zone between the individual cervix and the uterine horn, i.e. in those two regions which in the guinea pig seem to be especially sensitive as to atypical epithelial growth. We may recall here that atypical growth of the uterine epithelium even with metaplasia can be induced in the guinea pig (17) by a prolonged treatment with very small quantities of esterified estradiol just sufficient to maintain the uterus in the castrated female at a normal weight and still insufficient to induce fibroids. On the basis of our present experimental findings one must suppose that not only must treatment with estrogens be discontinuous so as to allow for a rhythmical diminution of the circulating estrogens in the body; diminution in the interval must be so considerable as to allow for a sinking of the folliculinemia beneath the threshold toxic for the uterine mucosa.

SUMMARY

The toxic effects of a prolonged treatment with estradiol monobenzoate have been studied in the female guinea pig under different timing conditions.

A given quantity of esterified estradiol which when injected thrice weekly, 47 injections in the course of 113 days, produces tumorigenic and other toxic effects (uterine bleeding, monstrous development of the uterus, uterine and extrauterine fibroids, polyps of the endometrium extending into the vagina) loses its toxic faculties if each week of injections is followed by 2 to 3 weeks free of injections (47 injections in the course of 365 days).

With these intervals free of injections quantities of esterified estradiol many times greater than the tumorigenic one are still unable to produce the above mentioned toxic effects.

Our thanks are due to Dr. K. Miescher of Messers. Ciba of Basle, Switzerland, for a very generous supply of estrogens.

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# PREVENTION OF EXPERIMENTAL UTERINE AND EXTRAUTERINE FIBROIDS BY TESTOSTERONE AND PROGESTERONE[1]

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ACCORDING TO LIPSCHÜTZ, VARGAS AND RUZ (1) uterine and extrauterine fibroids which are so easily induced by a prolonged treatment with estrogens in the guinea pig, can be partially prevented if testosterone is injected simultaneously with the tumorigenic quantities of estradiol. Lipschütz, Murillo and Vargas (2, 3) gave evidence that progesterone also is able to prevent these experimental fibroids. The antitumorigenic action of testosterone became manifest when the proportion of the esterified estradiol and the esterified testosterone was no less than 1:22; in other experiments proportions of 1:50 and more were made use of. The preventive action of progesterone was observed with quantities of the latter 150 times greater than those of estradiol. On account of the small number of our experiments and of the considerable individual variations as to the degree of the conjunctive tumoral reaction we raised the objection that the results may have been due to chance. But on the other side we stated that simultaneous injection of testosterone counteracts in the guinea pig the atypical growth of the endometrium due to estradiol. Also Korenchevsky and Hall (4) found that the atypical growth of the endometrium due to estrogens was prevented in the rat when injecting progesterone simultaneously.

Because of the great importance the statement of an eventual antitumorigenic action of testosterone and progesterone may have for the theory of fibromatogenesis and for treatment we decided to repeat our experiments with a greater number of animals. The preventive action of the two mentioned hormones against fibroids only shall be treated briefly in this paper based on 83 new experiments; the condition of the endometrium shall be dealt with later on.

### *Simultaneous Injection of Estradiol and Testosterone*

Twenty female guinea pigs (390 to 650 gm.), castrated at an early age, received thrice weekly subcutaneous injections of 40 to 80γ(0.04 to 0.08 mg.) of estradiol monobenzoate; 24 castrated guinea pigs (340 to 740 gm.) were injected simultaneously with estradiol monobenzoate and testosterone propionate the quantity of the latter being 50 times that of the estradiol, i.e., 2 to 4 mg. per injection. Postmortem examination was made 30 to 93 days after the beginning of treatment. All timing conditions were identical for both groups of animals. Special attention was given to the condition of the vagina which opens after the first injection of so considerable quan-

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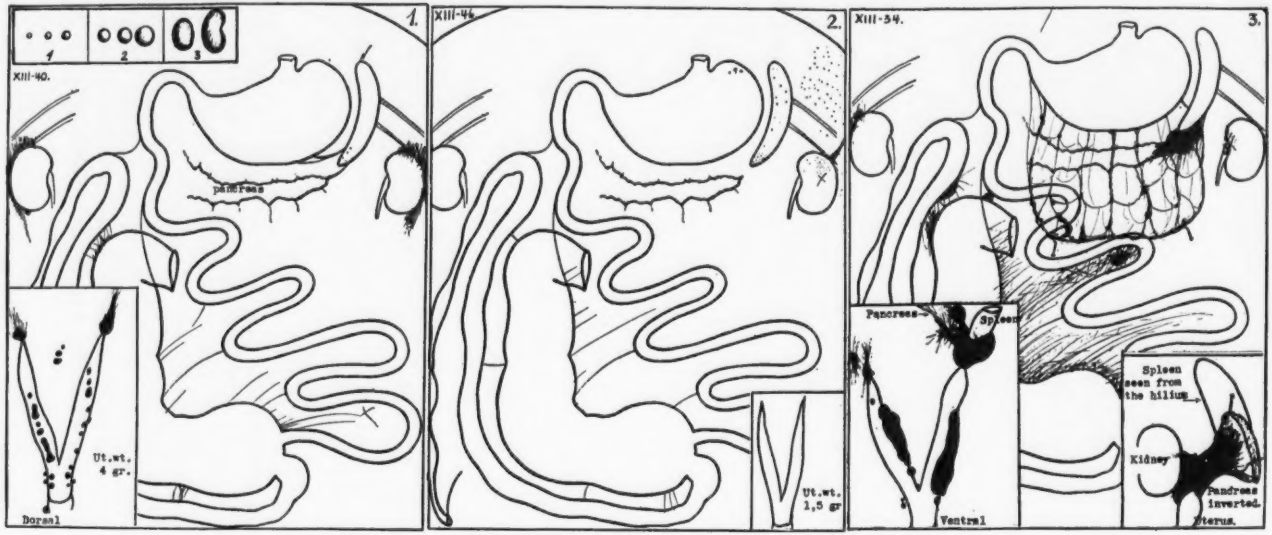


Fig. 1. AVERAGE FIBROUS REACTION IN ANIMAL TREATED WITH INJECTIONS OF ESTRADIOL ONLY. Castrated guinea pig, 0.08 mg. of estradiol monobenzoate thrice weekly for 30 days; total 0.96 mg. Multiple mesometrial tumors. Several small subserous uterine tumors and subserous tumor of the vagina (uterine tumoral mark, 2). Apical uterine tumors on both sides (apical mark, 2). Fibrous strands in the mesentery and around the kidneys; small subserous tumor beneath the right kidney; small subserous tumors of the abdominal wall (abdominal mark, 1). Tumoral seed on the under pole of the spleen and fibrous strands, between spleen and stomach (splenic mark, 0.5). Total tumoral mark, 5.5. Above on the left: size of tumors corresponding to marks 1 to 3. Fig. 2. MAXIMAL FIBROUS REACTION OF ANIMALS TREATED WITH INJECTIONS OF BOTH ESTRADIOL AND TESTOSTERONE. Eight hundredths of a mg. of estradiol monobenzoate and 4.0 mg. of testosterone thrice weekly for 63 days, total quantity of estradiol, 2.24 mg., and of testosterone, 112 mg. No uterine tumors. Tumoral seed on the spleen (splenic mark, 0.5); tumoral seed on the abdominal wall, stomach and left kidney some fibrous strands in the mesentery (abdominal mark, 0.5). Total tumoral mark, 1. Fig. 3. MAXIMAL FIBROUS REACTION IN ANIMAL TREATED WITH INJECTIONS OF ESTRADIOL ONLY. Eight hundredths of a mg. of estradiol monobenzoate thrice weekly for 63 days; total 2.0 mg. Enormous mesometrial tumors (mark, 3). Big apical tumor of left uterine horn and smaller apical tumor of right horn (apical mark, 3). Several mesenteric tumors, tumors of the epiploon and in other parts of the abdominal cavity; considerable fibrous strands in the mesentery and especially in the epiploon (abdominal mark, 2). Left apical tumor confluing with tumors of the spleen (splenic mark, 2) and of the pancreas. Total tumoral mark, 10.

tities of estradiol. The comparative results as to the vagina were overwhelming; it remained open in all the animals of the group treated with estradiol only, whereas in the group receiving both estradiol and testosterone the vagina became closed again after several injections and remained so for the whole time of combined treatment up to 3 months with only rare exceptions. Development of teats was not inhibited by the simultaneous administration of testosterone.

In one animal of the estradiol group which died 30 days after beginning of treatment (12 injections of 0.08 mg. each) multiple uterine tumors were found at postmortem examination; uterine and extrauterine fibroids were found in a second animal 36 days after beginning of treatment (15 injections of 0.08 mg. each). The total tu-

TABLE 1

| Exp. | No. of animals | Duration of exp. | Treatment | | Animals with some tumoral or fibrous reaction | Animals with individual tumors of mark not less than 1 | Fibroids and macroscopical fibrosis total mark | | Weight of uterus | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Estradiol/ week | Testostirone[1]/ week | | | Average | Range | Average | Range |
| | | days | mg. | mg. | | | | | gm. | gm. |
| Ia | 2 | 30–36 | 0.24 | 0 | All | All | 4.7 | 4–4.5 | 4.0 | 2.9–5.7 |
| Ib | 8 | 63 | 0.24 | 0 | All | All | 4.7 | 1.5–10 | | |
| IIa | 15 | 54–63 | 0.24 | 12.0C | 9 of 15 | None | 0.4 | 0–1 | 2.4 | 1.5–3.7 |
| Ic | 5 | 73 | 0.12 | 0 | 4 of 10 | 3 of 10 | 4.4 | 0.5–9 | 3.9 | 3.0–4.5 |
| Id | 5 | 91–93 | 0.12 | 0 | 4 of 10 | 4 of 10 | 3.1 | 0–5.5 | | |
| IIb | 2 | 66 | 0.12 | 6.0R | 2 of 9 | None | 0.5 | 0.5 | 2.0 | 1.3–2.5 |
| IIc | 3 | 73 | 0.12 | 6.0R | 2 of 9 | None | 0.3 | 0–0.5 | | |
| IId | 4 | 91 | 0.12 | 6.0C | 3 of 9 | None | 0.6 | 0–1 | | |
| Total: Estradiol (I) | 20 | 30–93 | 0.12–0.24 | 0 | 18 | 17 | 4.1±0.68 | 0–10 | 4.0[2]±0.74 | 2.9–5.7 |
| Estradiol and testosterone (II) | 24 | 54–91 | 0.12–0.24 | 6.0–12.0 | 16 | None | 0.4±0.07 | 0–1 | 2.2±0.43 | 1.3–3.7 |
| Estradiol and progesterone | 7 | 63 | 0.24 | 36.0 | 5 | None | 0.6±0.17 | 0–1 | 2.0±0.19 | 1.5–2.5 |

[1] C—Ciba. R—Laboratoires du Docteur Roussel.
[2] Only 13 animals with uterine weights; the remaining mostly of great development with tumors, kept for microscopial examination.

moral marks (see our system of marks for experimental fibroids (5), and explanations to figures of the present paper) were in both these cases 4 to 5 (fig. 1). In the group treated during 63 to 93 days the total tumoral marks were 4 to 10 in 8 of 18 animals (see *Ib*, *Ic* and *Id*, table 1); the average total mark in the 20 animals of the estradiol group was more than 4. For more details see table 1.

In the group of animals receiving the combined treatment of estradiol and testosterone not one animal of the 24 showed uterine fibroids. Sixteen out of these animals had some fibrous reaction in the mesentery or on the abdominal wall and the spleen. But this reaction was not considerable; it never surpassed the degree of what we termed 'tumoral seed' which occurs on the spleen and the surrounding parts of the stomach and the abdominal wall and is, according to the findings of Vargas and Lipschütz (6), the first manifestation of a conjunctive tumoral reaction induced by estrogens. The maximal reaction in the group receiving estradiol and testosterone with a total tumoral mark of 1 is shown in figure 2 which must be compared both with figure 1 showing the average, and with figure 3 showing the maximal reaction in the estradiol group. Of the 24 animals of the estradiol-testosterone group there was not a single animal with individual tumors of mark not less than 1 which were present in 17 out of 20 animals of the estradiol group.

When comparing the average total tumoral mark in both groups (4 and 0.4 respectively) it becomes evident that no less than 90% of the tumorigenic action of the

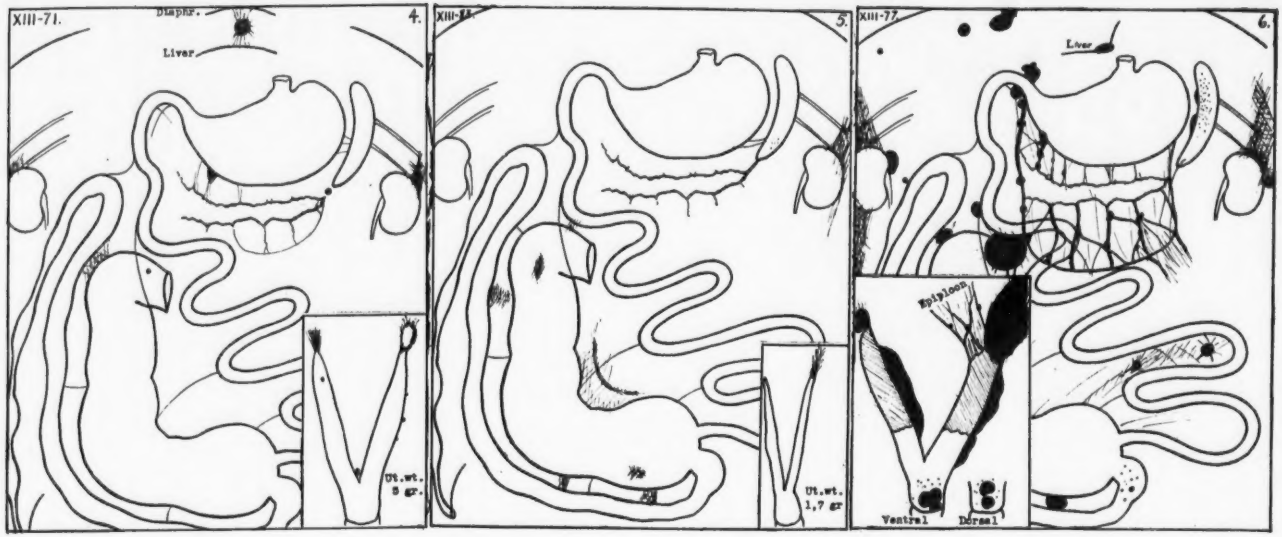


Fig. 4. SOMEWHAT LESS THAN THE AVERAGE FIBROUS REACTION IN ANIMAL WITH TABLETS OF ESTRADIOL ONLY. Three mg. of estradiol absorbed in 50 days from 37 mg. tablet. Small uterine and mesometrial tumors (uterine mark, 1). Apical fibrous strands especially around cystic tube (apical mark, 0.5). Tumor of great curvature of the stomach, tumor between pancreas and spleen; tumor in the ligament between diaphragm and liver, and others (abdominal mark, 2). Total tumoral mark, 3, 5. Fig. 5. MAXIMAL FIBROUS REACTION IN ANIMAL WITH TABLETS OF BOTH ESTRADIOL AND PROGESTERONE. Three mg. of estradiol absorbed in 45 days from 10 mg. tablet. Quantity of progesterone absorbed, 35 mg., from 2 tablets of 109 mg. total wt. No uterine tumors. Fibrous strands on the left uterine horn (apical mark, 0.5). Fibrous strands at different places of the mesentery and in other parts of the abdominal cavity (abdominal mark, 0.5). Tumoral seed on the spleen (splenic mark, 0.5). Total tumoral mark, 1.5. Fig. 6. MAXIMAL FIBROUS REACTION IN ANIMAL WITH TABLETS OF ESTRADIOL ONLY. Two and five-tenths mg. of estradiol absorbed in the course of 50 days from 7.5 mg. tablet. Subserous uterine and mesometrial tumors (uterine mark, 3). Big apical tumors on the left and smaller on the right horn (apical mark, 3). Many tumors in different parts of the abdominal cavity including diaphragm, liver epiploon, kidneys, abdominal wall, mesentery, pronounced fibrous strands of the epiploon (abdominal mark, 3). Tumoral seed on the surface and tumors in the hilum of the spleen (splenic mark, 2). Total tumoral mark, 11. There was in this case purulent peritonitis with uterine localization; see striped part of uterine horns. Adhesions of the epiploon to the left uterine horn.

esterified estradiol was suppressed or prevented by the simultaneous administration of esterified testosterone. The same is shown by the fact that the tumoral reaction in the group receiving simultaneously 0.04 mg. to 0.08 mg. of esterified estradiol and 2.0 and 4.0 mg. of esterified testosterone per injection in the course of 2 to 3 months was much smaller than with 0.005 mg. of esterified estradiol alone per injection.

Increase of uterine growth due to estradiol also was considerably diminished by the simultaneous administration of testosterone (table 1).

It is remarkable that testosterone did not suppress growth of the mammary gland. The clitoris hypertrophied and transformed into a hypospadic peniform organ as described 25 years ago by the senior writer from the laboratory of Steinach, in a female with testicular graft (7) (see also the work of Sand in the rat, 8). The growth of the clitoris was not suppressed by the simultaneous administration of estradiol

TABLE 2

| Exp. | No. of animals | Duration of exp. | Treatment | | Animals with some tumoral or fibrous reaction | Animals with individual tumors of mark not less than 1 | Fibroids and macroscopical fibrosis total mark | | Weight of uterus | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Wt. of tablets of estradiol | Wt. of tablets of progesterone | | | Average | Range | Average | Range |
| | | *days* | *mg.* | *mg.* | | | | | *gm.* | *gm.* |
| Ia | 6 | 45–50 | 6.0–13.0 | 0 | All | All | 4.1 | 1.5–11 | 4.0 | 2.5–6.2 |
| IIa | 7 | 45–50 | 7.5– 9.5 | ± 55 | 3 of 13 | None | 0.3 | 0–1 | 1.9 | 1.7–2.5 |
| IIb | 6 | 50 | 8.0–15.0 | 106–109 | 4 of 13 | None | 0.7 | 0–1 | 2.0 | 1.7–2.4 |
| Ib | 5 | 50–52 | 19.0–37.0 | 0 | All | All | 4.5 | 2–7 | 5.3 | 3.0–8.0 |
| IIc | 6 | 52 | 18.0–22.5 | 106–109 | 3 of 8 | None | 0.5 | 0–1 | 1.8 | 1.5–0.23 |
| IId | 2 | 50 | 35.0–38.5 | 214–218 | None of 8 | None | None | 0 | 1.8 | 1.8 |
| Total: Estradiol (I) | 11 | 45–52 | 6.0–37.0 | 0 | All | All | 4.2±0.85 | 1.5–11 | 3.4[1]±0.87 | 2.5–8.0 |
| Total: Estradiol and Progesterone (II) | 21 | 45–52 | 7.5–38.5 | 55–218 | 10 of 21 | None | 0.5±0.11 | 0–1.5 | 1.9[2]±0.23 | 1.5–2.4 |

[1] Only 7 animals with uterine weights; the remaining kept for microscopic examination.
[2] Only 20 animals with uterine weights.

under the present quantitative conditions, contrary to what we observed in former work of Ruz (9) in which smaller quantities of testosterone were given simultaneously with estradiol.

### *Simultaneous Injection of Estradiol and Progesterone*

Seven castrated female guinea pigs (260 to 440 gm.) received thrice weekly subcutaneous injections of 0.08 mg. of esterified estradiol (monobenzoate) for 2 weeks. Beginning with the third week daily injections of 6 mg. of progesterone were given besides continuing with the injections of estradiol as before; in this way the animals received 0.24 mg. of estradiol and 36 mg. of progesterone weekly. The vagina, which opened during treatment with estradiol, closed again in all the animals a few days after beginning treatment with progesterone. Postmortem examination was made 63 days after beginning treatment with estradiol and 48 days after beginning treatment with progesterone. There was no animal with uterine tumors, only tumoral seed on the spleen and the surrounding parts of the stomach and abdominal wall having been induced in 4 out of 7 animals. The maximal tumoral mark was 1 and the average tumoral mark 0.6 (see last line of table 1). The results may be compared with the estradiol group Ib of table 1 with an average tumoral mark of 4.7. Increase of uterine weight due to estradiol also was less.

*Implanted Tablets of Estradiol and Progesterone*

In the preceding experiments the proportion of estradiol and progesterone was of 1:150. It was thought that lesser quantities of progesterone may be also active in preventing fibroids when using the free estrogenic hormone instead of the esterified one. It might be recalled here that according to our findings (10, 11) the tumorigenic faculty of the follicular hormone is greatly enhanced by esterification. But with tablets of free estradiol introduced beneath the skin by the technic of Deanesly and Parkes (12, 13), Lipschütz and Vargas (14) were able to induce considerable development of fibroids in the course of 7 weeks. Will tablets of progesterone when introduced simultaneously with tablets of estradiol prevent formation of fibroids? And if so, which quantity of progesterone will be necessary? Our results with tablets are given in table 2.

The vagina which opened 1 to 5 days after introducing a tablet of estradiol beneath the skin opened also in most animals with both estradiol and progesterone but closed again in all of them and remained so for the rest of the experiment.

There was an average total tumoral mark of 4.2 (fig. 4) with tablets of estradiol, total tumoral marks ranging between 1.5 and 11 (fig. 6). On the contrary, there was no animal with individual tumors of mark 1 with tablets of both estradiol and progesterone (fig. 5). Ten of the latter 21 animals showed a tumoral seed on the spleen and the surrounding parts or some fibrous strands in the abdominal cavity. The total tumoral mark never attained more than 1.5 and the average was 0.5. There was no animal with uterine fibroids.

Uterine growth also was considerably less in animals with tablets of both estradiol and progesterone (table 2).

The total quantity of estradiol absorbed was 1 to 5 mg. in the course of 45 to 52 days, that of progesterone 18 to 48 mg. This is about 13 to 26% per month and gives full corroboration to the statement of Warwick and Parkes (15) who found in the guinea pig an absorption of 15 to 27% per month from tablets of progesterone weighing 38 to 57 mg. The average proportion between estradiol and progesterone absorbed was 1:13. In 14 out of 20 experiments the proportion was between 1:9 and 1:20; in 4 experiments the proportion was less and only in 2 more. All these data are not exact since desiccation of tablets was made at body temperature; but the error cannot be considerable.

## DISCUSSION

Our experiments with testosterone and progesterone leave no doubt as to the fact that both these hormones are able to prevent development of fibroids due to estradiol. Treatment with tablets of progesterone was shown to be very favorable when considering the quantities of progesterone necessary to prevent fibroids. We have not yet studied the same question for testosterone, but it seems very likely that tablets of testosterone also will be an advantage. Though all questions of clinical application are outside our field, according to our feeling the experimental statement that fibroids can be prevented by relatively small quantities of progesterone when using subcutaneous tablets, opens a new field of application for this ingenious technic introduced by Deanesly and Parkes.

## SUMMARY

Experimental uterine and extrauterine fibroids due, in the guinea pig, to a prolonged treatment with estradiol (31 experiments) were prevented by administering testosterone or progesterone simultaneously with the estrogen (52 experiments).

Uterine fibroids were completely prevented by injecting quantities of testosterone propionate 50 times greater or by injecting quantities of progesterone 150 times greater than those of the esterified estradiol.

Complete prevention of uterine fibroids was also obtained in experiments in which tablets of progesterone were implanted under the skin simultaneously with the tumorigenic tablet of free estradiol.

In the experiments with tablets the average proportion of absorbed estradiol and progesterone sufficient to prevent fibroids was of 1:13 only.

The extrauterine tumoral reaction due to estradiol was prevented to a very considerable degree (about 90% of prevention).

Our thanks are due to Dr. K. Miescher of Messers. Ciba in Basle, Switzerland, for a very liberal supply of estradiol monobenzoate and of testosterone propionate, to Laboratoires du Docteur Roussel in Paris for testosterone propionate and to Dr. M. Tausk of Messers. Organon in Oss, Holland, for progesterone.

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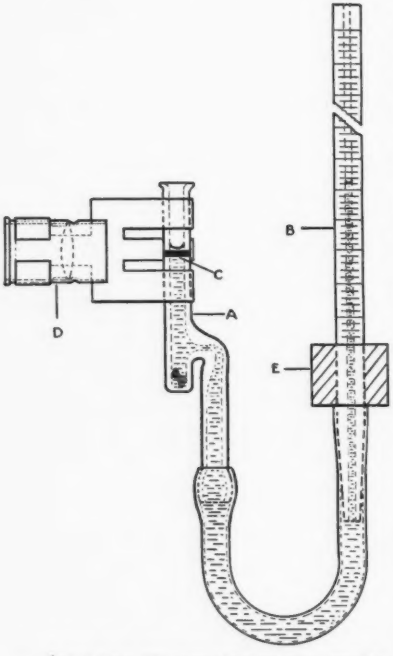
# NOTES AND COMMENTS

## A VOLUMETER FOR SMALL ORGANS

THE DETERMINATION of the volume of small organs has been something of a problem because some of the methods were particularly tedious, while others included serious sources of error. In the last few years, the writer has been using a volumeter which is simple yet accurate.




APPARATUS FOR DETERMINING THE VOLUME OF SMALL ORGANS. *A*—receiving tube; *B*—leveling pipette; *C*—black band for sighting across meniscus; *D*—burette meniscus magnifier; *E*—cork insulator.

The accompanying diagram shows the apparatus. It consists of a narrow, glass receiving-tube (A), with a side arm connected by rubber tubing to a leveling-tube (B) which is a 0.2 cc. calibrated serological pipette, graduated in thousandths of a cubic centimeter. A normal saline solution is used as the fluid medium. Around the receiving-tube, above the side arm, is a narrow black band (C) to facilitate sighting across the bottom of the meniscus. The rear arm of an ordinary burette meniscus-magnifier (D) was painted white so that, when in position and side-lighted, the meniscus can be sharply outlined on the upper edge of the black band (C). The receiving tube is fixed in position, but the leveling pipette is arranged to be held in a clamp by friction only, so that it can be shifted vertically. The meniscus in the leveling pipette is more easily seen if a sheet of white paper is held back of the pipette, with the paper back-lighted.

Before placing anything in the fluid in the receiving tube, the meniscus is brought sharply in line across the top of the black band; then the meniscus in the leveling pipette is observed and recorded. After an organ or gland is carefully inserted into the fluid of the receiving tube, the meniscus in this tube is again returned to its former position by lowering the leveling pipette. The difference in the readings of the two levels in the pipette is, of course, the volume of the organ.

With this apparatus, no attention is given to the temperature of the saline solution, which is that of the room, because these measurements are made so quickly that there is little opportunity for sudden expansions or contractions of the fluid in the pipette. However, sudden drafts and the heat from accessory lamps should be avoided. During the leveling process, the pipette may be protected from the heat of the fingers by a ring of cork (E) around its lower end.

In filling the apparatus with the saline solution, particular care is used to prevent the formation of air bubbles in both the tubing and the pipette. During measurements, excessive changes in the levels of the fluid are avoided so that capillary effects may be at a minimum. Frequently, when measuring several organs, such as the ovaries of mice which are not to be preserved for other purposes, it is customary to leave them in the receiving tube until all have been measured. This procedure reduces the number of intermediate readings. Furthermore, much of the accuracy of volume readings depends upon the observer's ability to properly and uniformly sight the meniscus in the receiving tube.

To remove the organs, a long stiff needle with a short, bent and very sharp point may be used. The advantage of the side arm on the receiving tube will be seen in the ease of removal of organs from the fluid. No organs can be lost down the rubber tubing, nor can they prevent the leveling process.

For practical purposes, receiving tubes of different diameters are used, depending upon the size of various organs to be measured. In measuring the volumes of such small organs as the ovaries and adrenals of mice, it is our policy to measure both of a pair together.

The writer wishes to express his appreciation to Mr. Richard Cragg, formerly of the New York State College of Ceramics, who made the receiving tubes.

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# RESPONSE OF THE PREPUTIAL GLANDS OF THE FEMALE MOUSE TO TESTOSTERONE PROPIONATE

SINCE KORENCHEVSKY AND DENNISON[1] in 1936 described the hypertrophy of the preputial glands of the female rat following injections of testosterone and androsterone, several other investigators[2,3,4,5,6,7] have noted similar results.

Our attention was first called to a similar hypertrophy in female mice by the difference in appearance of the pelvic region after a series of injections of testosterone propionate. These preputial glands were frequently so enlarged that the females superficially resembled males as they ran about in the cages. Even the gait and behavior were more like those of the male animal.

This investigation was undertaken to determine whether there was a proportional relationship between the response in growth of the preputial glands and the dosage of an androgen. It was found that, within certain limits, both the size (volume) and the weight of these glands are proportional to the amount of testosterone propionate injected.

*Procedure.* Young mature albino female mice were injected once daily with 2 mg. testosterone propionate* for 1 to 7 days. At autopsy, the weights and sizes of the ovaries, adrenals, preputial glands, and the weights of the spleens were recorded. Only the preputial glands will be considered at this time. The size of these glands was determined by the displacement of saline solution in a specially constructed volumeter for small organs.[8] The weights were

[1] KORENCHEVSKY, V., AND M. DENNISON: *J. Path. & Bact.* 42: 91. 1936; 43: 345. 1936.
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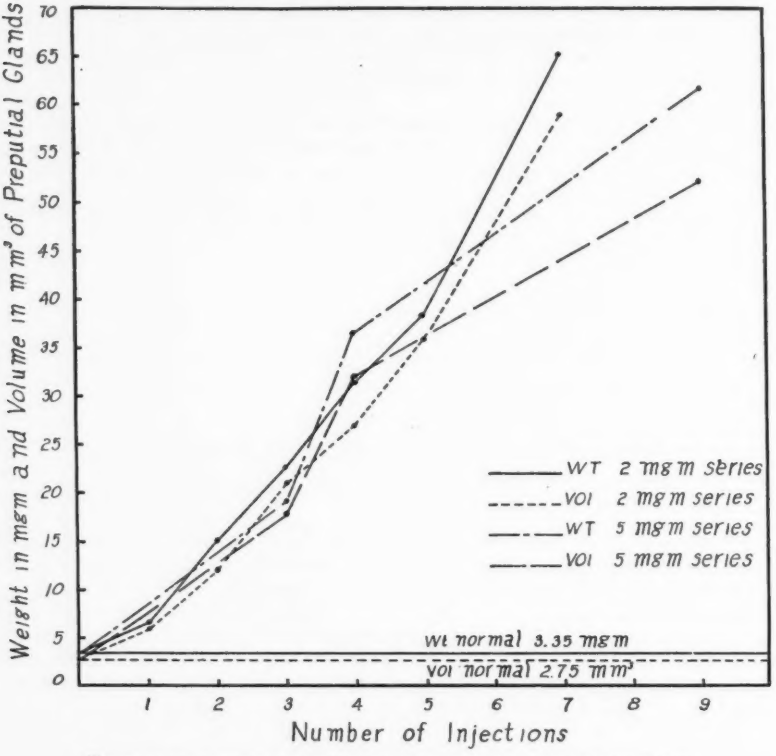
[7] SELYE, H.: *Canad. M. A. J.* 42: 113. 1940.
* Generous supplies of oreton (testosterone propionate) were furnished by Schering Corporation, through the courtesy of Dr. Gregory Stragnell.
[8] BURDICK, H. O.: *Endocrinology.* 28: 676. 1941.

obtained by means of an Ainsworth chainomatic balance. Both of a pair of glands were measured together as a unit. One small group was injected with 5 mg. for several days.

The average weight of both preputial glands from these young mature female mice was 3.35 mg., but after injections of testosterone propionate these glands averaged 65 mg. Their normal volume was 2.75 mm.$^3$ after 7 days the average was 59 mm.$^3$ Therefore the weight average had increased 28 times, and the volume 21 times.

The graph shows how closely the volume increase parallels that of weight, except that




EFFECT OF TESTOSTERONE PROFIONATE ON THE PREPUTIAL GLANDS OF FEMALE MICE.

there appears to be a greater spread between these two factors as the injections continue. This spread is particularly well shown in the 5 mg. series, in which, after 3 daily injections, the volume subtracted from the weight gave a factor of 1; after 4 injections, 4.4; after 9 injections, 9.2. These glands, after 9 injections, appeared to be firmer, and contained noticeably less of their usual sebaceous secretion.

The limit of response of these glands to testosterone propionate was not determined, but it is significant that the greatest weight recorded in the 2 mg. series was 65.0 mg. after 7 days, while the average after 9 daily injections of 5 mg. was 61.9 mg. However, one animal in this latter group had glands 81.7 mg. in weight and 70.0 mm.$^3$ in volume.

*Discussion.* Investigations on the responses of female preputial glands to androgens seem to have started with the work of Korenchevsky and Dennison,[1] who indicated that not only were they unable to find papers dealing with the effects of hormones on these glands, but that there seemed to be no description of the glands in the literature. Since they were similar in location, general appearance and histological structure to the preputial glands in the male rat, it was proposed by these investigators to call them the female preputial glands.

Therefore, these glands should not be called homologues of the prostate.[4] Actually, the female homologues of the prostate are the paraurethral glands of Skene.[2,9]

Just what function these preputial glands have in the female is not known, but they are particularly sensitive to injections of male hormone. Salmon[4] noticed their enlargement in immature female rats as early as 48 hours after the first injection. Control glands varied from 10 to 16 mg., but after testosterone propionate they had increased in weight to 35 to 88 mg. He showed, furthermore, that the ovary was not a necessary factor, for the preputials of castrates also enlarged. Korenchevsky, Dennison and Hall[10] injected normal adult female rats daily for 21 days with 0.5 mg. and 1.5 mg. testosterone propionate, then weighed various organs. The preputial glands of controls weighed only 103 mg., while those of the experimental series averaged 224 mg. and 256 respectively. It would have been of interest to have known whether, during the 21 days, these glands had reached a physiological limit of hypertrophy. At the end of the experimental period, they may have been decreasing in weight.

Several investigators have called attention to the fact that, as the glands hypertrophy, they become filled with a sebaceous fluid. Care must be exercised in the removal of glands, not to squeeze out this substance, as a loss of fluid will affect the weight-volume relationship. It may be of further interest to note that, as the injections continued over several days, the glands seemed firmer and contained less fluid. The weight-volume relationship was also altered. Just what effect, if any, these hypertrophied preputial glands have on other sexual organs or sexual responses of the female mouse is not yet known.

The fact that the female preputial glands are so susceptible to the influence of testosterone propionate but not to estrogens[1,2,7] suggest that they may be of use in the bioassay of androgens.

*Summary.* Daily injections of 2 mg. of testosterone propionate into young normal mature female mice caused hypertrophy of the preputial glands. Within 7 days the average weight of a pair of these glands had increased from 3.35 mg. to 65 mg.; the volume, from 2.75 mm.$^3$ to 49 mm.$^3$ This is an average weight increase of 28 times, and an average volume increase of 21 times.

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## SUBCUTANEOUS ADMINISTRATION OF INSULIN BY PELLETS

THE SUCCESSFUL ADMINISTRATION of testosterone, estrone, and desoxycorticosterone by the use of subcutaneously implanted pellets has suggested that insulin might be given in a similar manner. Mark and Biskind[1] tried pellets of both crystalline and protamine zinc insulin but found that absorption was fairly rapid, the maximum duration of the hypoglycemic effect not exceeding about 100 hours.

*Methods and results.* In an attempt to delay the absorption of insulin various substances were ground finely with it, and pellets made of the resulting mixtures. Cholesterol, cholesterol and lecithin, cholesterol and 'enterab'* (enteric coating), cholesterol and a shellac-castor oil enteric coating, charcoal and parlodion were used in varying proportions with crystalline zinc insulin.† The pellets were then implanted subcutaneously in the abdomen in normal fastened rabbits, and blood sugar estimations[2] made at appropriate intervals.

[1] MARK, J., AND G. BISKIND: *Endocrinology* 26: 444. 1940.
* Supplied by Abbott Laboratories, North Chicago, Illinois.
† Supplied by the Eli Lilly Company, Indianapolis, Indiana.
[2] FOLIN, O., AND H. MALINROS: *J. Biol. Chem.* 83: 115. 1929.

The two most promising mixtures were used in depancreatized dogs, maintained on a diet including raw pancreas. These were mixtures of equal parts of insulin and cholesterol, and insulin and cholesterol with a minute amount of lecithin. Pellets of the former could be made satisfactorily only in a hydraulic press, while the binding effect of the lecithin allowed the latter to be made in simple steel moulds by hammering. The hydraulic press pellets were kindly made for us by Dr. G. Biskind of the Mount Zion Hospital, San Francisco. The pellets were not sterilized. Autoclaved pellets were found to be ineffective. The individual pellets contained from 100 to 200 U of insulin, several being implanted simultaneously when larger doses were used.

Six dogs were used, which, when depancreatized, required from 10 to 20 U of crystalline zinc insulin daily to control their glycosuria. Brief protocols of these dogs follow.

*Dog 1* (5 kg.) remained sugar free, and with normal blood sugars for 13 days after the implantation of 200 U of insulin in pellet form. A second trial of the same dose resulted in death in hypoglycemia on the third day after implantation.

*Dog 2* (4 kg.) was controlled on one occasion for13 days by 150 U in pellets, and on a second occasion for 10 days by 150 U, at which time death resulted from pneumonia.

*Dog 3* (9 kg.) died in hypoglycemia the day after the implantation of 300 U in pellet form.

*Dog 4* (13 kg.) was controlled for only 7 days after the implantation of 400 U, and again for only 2 days after 300 U.

*Dog 5* (9 kg.) died in hypoglycemia 2 days after the implantation of 200 U of insulin.

*Dog 6* (14.5 kg.) suffered from severe hypoglycemia one day after the implantation of 500 U, and on a later trial, died in hypoglycemia 9 days after the implantation of 400 U.

Thus it is seen that there were periods of satisfactory control lasting as long as 13 days but that the chance for hypoglycemia was great, and at other times the effect was rather transient. These irregularities may probably be explained by disintegration or softening of the pellets, and the presence of scar tissue or infection around the pellets. The preparations did not fulfil the ideal of a slowly, but regularly, absorbable pellet which could be easily sterilized, and which would eventually be completely absorbed from the subcutaneous tissue. The results emphasize the danger in the introduction of any drug into the body in doses definitely exceeding the physiological range.

*Summary.* Pellets of cholesterol and insulin, implanted subcutaneously in depancreatized dogs, gave a slow release of insulin during periods as long as 13 days. However, irregularities of absorption were too great to justify the trial of such pellets in clinical treatment of diabetes.

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